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A Bone Disorder Associated with Copper Deficiency.
I. Gross Morphological, Roentgenological and Chemical Observations. JAMES H. BAXTER AND JUDSON J. VAN WYK 1

II. Histological and Chemical Studies on the Bones.
JAMES H. BAXTER, JUDSON J. VAN WYK AND RICHARD H. FOLLIS, JR. 25

The Anemia of Copper Deficiency in Dogs Compared with That Produced by Iron Deficiency. JUDSON J. VAN WYK, JAMES H. BAXTER, JOSEPH H. AKEROYD AND ARNO G. MOTULSKY 41

The Filter Paper Electrophoretic Identification of Urinary Chorionic Gonadotrophin. A New Test for Pregnancy.
H. M. STRAN AND G. E. SEEGAR JONES 51

Proceedings of the Meeting of the Johns Hopkins Medical Society in Association with the Alpha Omega Alpha Fraternity, April 13, 1953. 54

Book Reviews 61

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A BONE DISORDER ASSOCIATED WITH COPPER DEFICIENCY

I. Gross Morphological, Roentgenological, and Chemical Observations

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In the course of a study in 1950, four weanling dogs after subsisting for several months on a diet of highly purified ingredients were observed to develop lameness and inability to stand. At the same time the dogs were found to be moderately anemic. It was believed that the lameness was a result of a neuromuscular disorder caused by an intercurrent infectious disease or by a dietary deficiency. A systematic search for a deficiency was undertaken. After the observation that no beneficial effects followed the administration of various pure vitamins or purified liver extract, it was discovered that a salt mixture had been employed which was deficient in trace elements.[1]

It became apparent in subsequent experiments that the lameness was the result of a bone disorder rather than a neuromuscular disturbance, and that the disorder could be produced by omitting copper alone from an otherwise adequate diet (1). Furthermore, the disorder was prevented, or alleviated after its occurrence, by the addition of copper. The disorder appeared to be a specific effect of copper deficiency, rather than the result of anemia or generally poor condition of the animals. Chemical studies afforded no evidence of a primary disturbance in calcium or phosphorus metabolism to account for the lesions. Observations on the animals, including the results of roentgenological, blood chemical, and morphological studies in the gross, are presented in the present report. The histological and chemical studies on the bones, and morphological observations on blood and bone marrow, are considered in the accompanying articles (2, 3).

METHODS

Animals.—Litters of weanling mongrel dogs were employed. The pups were separated from the mothers at the age of 3 to 5 weeks, divided into comparable groups, and placed on the experimental diets. In some experiments, cow's milk or other low copper diet was fed to all of the groups for one to several weeks during the period of transition from mother's milk to the final experimental diets. In each experiment, control animals were fed the deficient diet with copper added. The animals were maintained in metabolic cages constructed of galvanized iron. They were treated for worms and given injections of anti-distemper serum. Animals which developed diarrhea of more than a few days duration, or other significant illness of any type, were discarded.

* Now in the Department of Pediatrics, Johns Hopkins Hospital.

[1] The mixture employed was salt mixture No. 2, U.S.P. XIII, purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

Diets.—All diets were fed *ad libitum*. In experiments 1 to 11, purified diets of the following percentage composition were employed: casein 20, Crisco 19, corn oil 1, sucrose 55.7, salt mixture 4, and choline chloride 0.3. Vitamins were added per kg. of diet as follows: thiamine chloride 20 mg., pyridoxine 20 mg., riboflavine 30 mg., Ca pantothenate 50 mg., nicotinic acid 200 mg., inositol 500 mg., ascorbic acid 500 mg., para-aminobenzoic acid 10 mg., biotin 0.1 mg., folic acid 0.4 mg., vitamin B_{12} 60 μg., 2-methyl-1, 4-naphthoquinone 1.5 mg., dl-α-tocopherol acetate 15 mg., vitamin A 60,000 U.S.P. units and vitamin D 8,500 U.S.P. units.[2] Additional supplements of vitamin D, folic acid and vitamin B_{12} were administered in several experiments without apparent effect. The salt mixture employed in experiment 2 and in one copper-deficient group of experiment 3 was salt mixture No. 2, U.S.P. XIII. In the other experiments utilizing the purified diet, the salt mixture was a modification of Wesson's mixture (4), prepared in this laboratory from reagent-grade chemicals. The present modification was composed as follows: NaCl 107, KCl 120, KH_2PO_4 310, $Ca_3(PO_4)_2$ 150, $CaCO_3$ 210, $MgSO_4 \cdot 7H_2O$ 192, $FeCl_2 \cdot 4H_2O$ 15.6, $MnCl_2 \cdot 4H_2O$ 0.3, $Al(SO_4)_3 \cdot 18H_2O$ 0.1, NaF 0.5, KI 0.5, $ZnSO_4 \cdot 7H_2O$ 0.3, $Co(NO_3)_2 \cdot 6H_2O$ 0.3, $CuSO_4 \cdot 5H_2O$ 0.8–3.0. Copper was omitted from all of the copper-deficient diets.

The copper-deficient purified diets in most instances contained a little less than 1 μg. of copper per gram of diet. Most of this copper was derived from the casein. In experiment 8, the copper was reduced consistently to less than 0.5 μg. per gram of diet by reprecipitating the casein in the presence of disodium versenate. The control diet in the beginning contained 8μg. Cu per gram of diet. This was increased to 15 μg. per gram and finally to 30 μg. per gram because of the occurrence in control dogs of serum copper levels which were regarded as subnormal.

In experiment 12, the diet consisted of fresh cow's milk, with iron, the trace elements, and the vitamins, added in the same amounts on a dry-weight basis as in the later purified diet. In experiment 13, a diet of 85 per cent dried milk[3] and 15 per cent sucrose, with the same additions, was fed in the form of an agar gel. The latter diet was the more satisfactory of the two. The copper content of the modified milk diets was approximately the same on a water-free basis as that of the purified diets.

In experiments 11 and 13, the effects of copper deficiency were compared with the effects of iron deficiency. The iron-deficient groups were fed the same levels of copper as the controls, but greatly reduced amounts of iron. In experiment 11, the iron content of the iron-deficient diet was varied from none at all to one-fourth of the control level; in experiment 13 no iron was added to the diet of the iron-deficient group.

Water demineralized by passage through exchange resins and containing less than 1 μg. Cu per liter was utilized in all experiments.

The approximate average calcium and phosphorus contents of the various diets on a dry-weight basis were as follows:

	Ca PER CENT	P PER CENT	Ca/P
Purified diet with salt No. 2, U.S.P. (Exp. 2 and one group of Exp. 3)	0.32	0.52	0.6
Purified diet with modified Wesson's mixture (Exps. 3–11)	0.61	0.55	1.1
Raw Milk Diet (Exp. 12)	0.93	0.70	1.3
Dried Milk Diet (Exp. 13)	0.79	0.57	1.4

[2] Vitamins A and D usually were given as Oleum Percomorphum, obtained from Mead Johnson & Co., Evansville, Ind., though crystalline vitamins were used in one experiment.

[3] Klim, purchased from Borden Food Products Company, New York, N. Y.

Methods of Observations.—The dogs were weighed and examined outside of the cages at weekly intervals. X-ray examinations of the fore and hind legs were performed at intervals of several weeks.

Copper determinations on diets, diet ingredients, liver, and other biological materials with the exception of serum, were carried out by digestion, isolation of copper with dithizone, and determination of copper with diethyldithiocarbamate, as described by Sandell (5). The method of Cartwright *et al.* (6) was employed for the copper determinations on serum. Inorganic phosphorus in serum was determined by the method of Fiske and SubbaRow (7), and alkaline phosphatase was determined by the method of Bodansky (8), utilizing the Fiske-SubbaRow method for inorganic phosphorus. Serum calcium was determined by the Clark-Collip modification of the Kramer-Tisdall method (9). Serum vitamin D was estimated by bioassay in rachitic rats (10).

After 3 to 5 months on the experimental diets, some of the animals were treated with copper and the remainder were autopsied. The limb bones, ribs, and sometimes vertebrae of the autopsied animals were cleaned, split with a scroll saw, and washed out in a jet of water. Corresponding bones of controls and deficient animals were then compared side by side. Samples of tissues were prepared for chemical and microscopic examinations.

RESULTS

Observations on the Bone Disorder

After periods of 2 to 4 months on the copper-deficient diets, more than two-thirds of the animals were observed to develop lameness or deformities of the extremities (Figs. 1–5). The forelegs were bowed outward at the elbows, while the hind legs were bowed inward with the knees held together for support. The ends of the bones about the wrists and elbows often became large and "knotty." Hyperextension of the wrist joints was a prominent feature in some experiments, leading to a decrease in height of the anterior end of the animal and giving the animal the appearance of walking on snowshoes. A number of animals developed obvious fractures, sometimes multiple fractures, of the limb bones. The fractures occurred in most cases without known cause, or sometimes as a result of mild stress such as that encountered in restraining the animal for venipuncture. Characteristic bone changes were observed in the deficient animals in all of the experiments, though the deformities were more severe on the purified diets than on the milk diets. The differences with the different diets may have been due to chance. In all cases, the control animals appeared to be normal. The observations on the bone changes and associated symptoms occurring in the dogs as a result of copper deficiency are summarized in Table I.

Atrophy of the muscles of the hind quarters and tenderness about the extremities, probably secondary to bone alterations, were observed in some animals. There appeared to be no changes in reflexes or in sensory perception. On microscopic examination, sections from various portions of the central and peripheral nervous system appeared normal, as did sections of muscles.[4]

[4] For the preparation of these and various other sections, and for help in their interpre-

It was evident from comparisons of roentgenograms and of bones themselves, that the bones of the copper-deficient animals were characterized by a decrease in thickness of the cortices (Figs. 6–14). This change was observed most consistently in the shaft of the femur, but it was prominent also in the metacarpals and other limb bones. The decrease in thickness of cortical bone in the deficient animals was associated with an increase in width of the medullary cavity and an apparent increase in volume of the marrow. The marrow was pale and contained less fat than normal.

Another consistent abnormality of the bones of the deficient animals was a deficiency of trabeculae. Trabeculae often were lacking throughout the diaphyses, and the metaphyses were of diminished extent. In addition, the tra-

TABLE I

*Summary of Results**

DIET†	NO. OF DOGS	PLASMA COPPER	NUMBER OF DOGS WITH FOLLOWING FEATURES						
			Graying of hair	Hematocrit			Lameness or deformity of legs	Fractures	Thin bones and deficient trabeculae
				8–20%	21–39%	40–55%			
Complete	19	84 (52–108)	0	0	1	18	0	0	0
Copper-deficient	27	28 (17–38)	19	6	19	2	19	9	20 (24)‡
Iron-deficient	5	64 (52–74)	0	2	3	0	0	0	0

* The results are tabulated on the basis of observations made at the height of symptoms in the deficient animals, near the end of the experimental periods.

† Results from experiments utilizing both purified and modified milk diets are included in this table.

‡ Four lame dogs from an early experiment were not examined completely.

beculae of the metaphyses of the limb bones, of the epiphyseal centers of ossification, and of such bones as the ribs and vertebrae were more delicate, of smaller caliber, and more widely spaced than normal (Fig. 14). This deficiency of trabeculae was partially responsible for the "ground-glass" appearance of the bones in roentgenograms. This latter alteration was most readily visualized in the centers of ossification of the distal radial epiphyses and other small bones about the wrists (Fig. 7), and in the femurs (Fig. 10).

The bones of the copper-deficient animals were as mature as those of the controls, judging on the basis of maturation of centers of ossification of the epiphyses. Furthermore, the limb bones of the deficient animals were as large or larger in external dimensions than those of corresponding control animals.

tation, we are indebted to Dr. James H. Peers of the Section of Pathological Anatomy, NIAMD, NIH.

After they were freed of soft tissue and marrow, bones of the deficient animals were abnormally pale, due apparently to the presence of fewer vessels and less blood in the bone structure. The epiphyseal cartilages of the copper-deficient animals were increased in thickness.

In addition to these basic alterations in the bones of deficient animals, there were a number of changes which were interpreted as effects of stresses acting on abnormally weak bones. These changes included bowing, compensatory thickening, fractures, displacement and distortion of epiphyses, and longitudinal compression with lateral expansion of the ends of limb bones (Fig. 8). This last change probably accounted for the enlargement and hyperextension of joints. The lameness which has been described was a result of these various secondary alterations.

Other Symptoms Associated with Bone Alterations

The hair of many of the copper-deficient dogs became gray, rough and dull. Since some normal dogs exhibit graying, the hair changes were relative, and they were seen most readily in dogs with dark hair. The alterations appeared first about the eyes and nose. When generalized, the graying was prominent about the neck and in areas that had been clipped. The hair changes sometimes developed before, or in the absence of, lameness and other symptoms of the deficiency. Roughness and scaling of the skin occurred in most of the animals with prominent hair changes. The epidermis of these animals appeared thin and was covered by a loose mantle of keratin scales.

There appeared to be a distinct correlation in the copper-deficient animals between the severity of the bone alterations and the degree of anemia. The two alterations made their appearance at about the same time, and definite bone changes were not observed in the absence of some degree of anemia. These observations may have indicated simply that the two disturbances were caused by the common factor of copper deficiency.

The hemoglobin levels per 100 ml. of blood in the weanling pups often were as low as 6 to 9 grams. In both controls and copper-deficient animals, the levels in most cases rose by the end of the first month on the diets to the neighborhood of 12 grams. At this stage, if not earlier, differences appeared between the two groups in the trends of hemoglobin and hematocrit values. While the levels in the controls continued upward to reach values of 15 to 16 grams after 2 or 3 months, the levels of many deficient animals first became stationary and then began to fall (Chart 1 and Table II). The anemia as a result of copper deficiency was accompanied by subnormal numbers of reticulocytes, normal or somewhat reduced numbers of leucocytes, and increased numbers of platelets.

The dogs ate the experimental diets readily and the rate of growth was fully equal to that on the kennel ration. At least half of the copper-deficient animals lagged somewhat behind the controls in weight gain (Chart 1 and Table II),

but the rate of growth in frame was approximately equal in the two groups. Although the dogs were confined to cages with limited space, they were able to

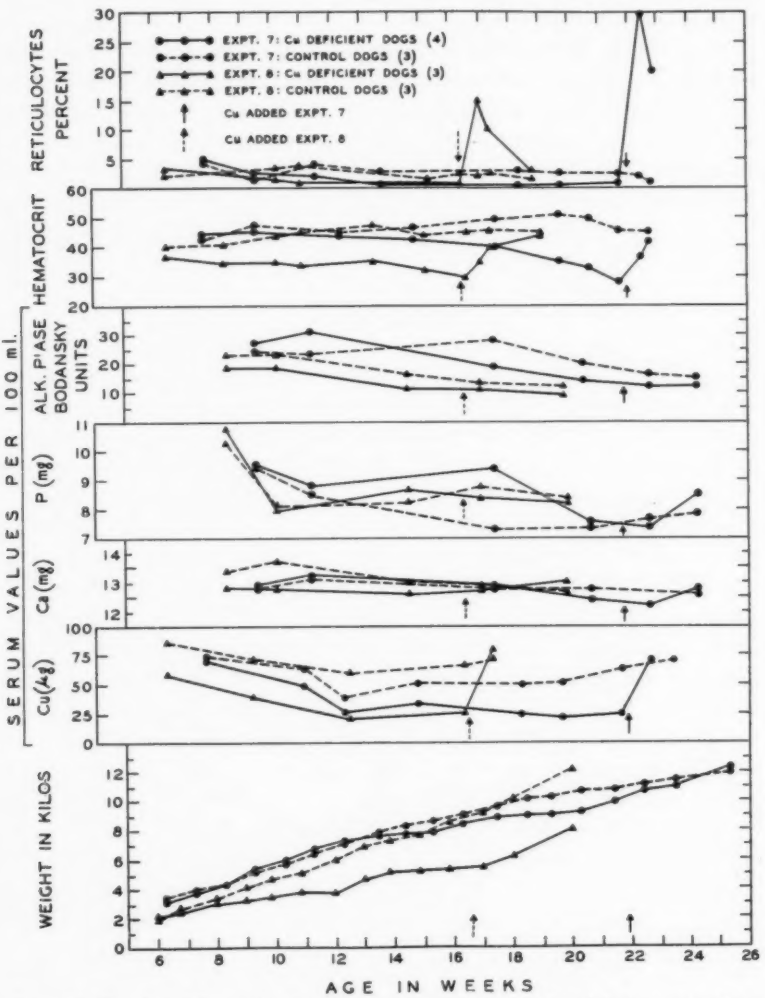

CHART 1. Results of determinations on dogs in experiments 7 and 8. Average values for each group are shown. The dogs were started on the purified diets with modified Wesson's salt mixture at the age of about 5 weeks.

play and jump about. Prior to the occurrence of severe lameness or extreme degrees of anemia, the deficient animals were as active and in as good health generally as were the controls.

The teeth of the deficient animals appeared on inspection to be normal. No pathological bleeding was noted in gums, mucous membranes, or skin. On inspection in the gross and on examinations of sections, the parathyroids and other endocrine glands appeared normal. A description of the bone marrow alterations is presented elsewhere (3). Small amounts of hemosiderin were present in the spleen and bone marrow. No abnormalities were noted in the remaining visceral organs.

TABLE II

Average Results of Determinations in Experiments 3, 9, 11, 12 and 13

EXP. NO.	DIET*	GROUP	NO. OF ANIMALS	WEIGHT GAIN†	SERUM‡					HEMATOCRIT‡
					Fe	Cu	Ca	Inorg. P	Alk. P'ase.	
				Kg.	μg. per 100 ml.	μg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	Bodansky units	Per cent
3	Purified	Control	3	7.15		85	12.0	7.6	20	46
		Cu-defic.	3	5.80		30	11.9	7.6	13	22
		Cu-defic. (Salt No. 2)	3	4.46		28	11.8	7.6	13	14
9	Purified	Control	3	6.68		98	12.7	9.5	18	43
		Cu-defic.	3	4.43		32	12.5	9.3	15	33
11	Purified	Control	3	9.61	104	78	11.3	8.8	19	48
		Cu-defic.	3	7.87	68	25	11.4	7.7	19	40
		Fe-defic.	3	8.68	58	62	11.6	7.7	26	37
12	Modified fresh milk	Control	2	6.64		102	12.9	7.5	6.2	49
		Cu-defic.	2	6.60		35	12.9	7.6	6.3	29
13	Modified dried milk	Control	2	8.25		85	11.3	8.5	7.8	40
		Cu-defic.	2	8.08		26	12.0	8.8	8.3	29
		Fe-defic.	2	8.26		62	11.1	8.3	7.7	20

* See text for complete description of diets. Except in the case of one group of Exp. 3, the purified diets contained the modified Wesson's salt mixture.

† During the experimental period of 3 to 5 months.

‡ The results on serum and blood were obtained during the latter part of the experimental periods near the height of the deficiencies. However, at the time of the determinations, the ages of the animals varied somewhat from one experiment to another.

Chemical Studies

Serum copper levels near 100 μg. per 100 ml. were observed in the majority of control animals, at least during the latter portions of the experiments (Table II). Distinctly lower levels in earlier periods of experiments 3 and 7 (Chart 1) probably were results of suboptimal levels of copper in the control diet during portions of these experiments. Even in these experiments, the serum copper levels of the controls were considerably above those of the de-

ficient animals, and none of the controls developed symptoms. Serum copper in the dogs on the copper-deficient diets fell gradually over periods of 1 month or more to levels of 20 to 35 μg. Levels above 40 μg. never were observed at the height of the deficiency, even in animals which did not develop bone changes. On the other hand, copper levels seldom fell much below 20 μg. per 100 ml., suggesting that a "floor" was reached.

Liver copper levels in control dogs at the ends of the experiments were 25 to 50 μg. per gram of wet tissue. The levels in corresponding deficient animals were in the range of 1 to 3 μg. per gram. The results are summarized in Table III. A few determinations of copper in bone, bone marrow, hair, and several other tissues showed distinctly lower levels in the deficient animals.

Serum calcium values were the same in controls and copper-deficient animals (Chart 1 and Table II). The levels varied from 11 to 14 mg. per 100 ml., with

TABLE III

Liver Copper Levels in Autopsied Dogs

GROUP	NO. OF DOGS	LIVER COPPER* μg/gm
Control	6	33.6 (24.0–42.9)
Fe-defic.	2	36.2 (35.4–37.0)
Cu-defic.	8	2.0 (0.9–3.0)
Cu-defic., treated	1	92.5

* Average values are given, with the range in parentheses. Determinations on each dog were done in duplicate.

the higher levels in the younger animals. A few semiquantitative tests on urine showed no great change in calcium excretion as a result of copper deficiency.

Inorganic phosphorus determinations on serum showed levels of 8.5 to 10 mg. per 100 ml. in young animals and 7 to 9 mg. in older animals. There was no consistent difference between controls and deficient animals.

Alkaline phosphatase determinations on serum in all experiments utilizing purified diets afforded results ranging from 10 to 43 Bodansky units, with most of the values between 15 and 30 units. The higher results were obtained in the younger animals, and there was some decrease in levels with increasing age. In experiments 12 and 13, in which the animals were on supplemented milk diets, alkaline phosphatase levels between 5 and 10 Bodansky units were observed. These latter values corresponded to those observed in a few similar animals under "normal" conditions on the outside. In some of the experiments, the phosphatase levels were slightly lower in copper-deficient animals than in controls. However, these differences were not considered significant. The re-

sults in experiments 12 and 13 indicated that high alkaline phosphatase levels of the earlier experiments were not essential for the development of the characteristic bone lesions of copper deficiency.

Assays of plasma from two copper-deficient dogs with severe bone lesions for vitamin D content showed normal levels of approximately 100 U.S.P. units per 100 ml. Ascorbic acid levels of serum and whole blood in two deficient animals also were observed to be normal. Potassium, carbon dioxide, and pH of arterial plasma in one experiment were the same in controls and deficient animals; the average values were respectively 4.85 meq./l., 20.7 meq./l., and 7.28.

Effects of Therapy

The addition of copper sulfate alone to the food and drinking water of the copper-deficient dogs was followed by prompt and often dramatic improvement. Reticulocytes increased, reaching levels of 20 to 50 per cent within 4 to 6 days in severely anemic dogs. Hemoglobin levels returned approximately to normal within about 3 weeks. General appearance and activity improved within a few days. Following an initial increase in scaling of the skin of some animals, the hair and skin returned gradually to normal. No further fractures occurred after beginning therapy. The radiographic appearance of the bones became normal after 6 weeks to 2 months, though some of the deformities were not corrected completely. The copper content of plasma and liver increased promptly. There was no doubt that all of the symptoms in the deficient animals were specifically relieved by the administration of copper.

Specificity of Bone Alterations Due to Copper Deficiency

It has been concluded by other investigators that the morphological and chemical changes in peripheral blood and bone marrow resulting from copper deficiency are in most respects similar to those produced by iron deficiency (11), and that copper-deficiency anemia actually is the result of defective absorption and utilization of iron (12). Thus, on the assumption that the effects of copper deficiency on blood and bone marrow are reproduced by iron deficiency, it seemed that iron deficiency might afford a means of distinguishing between primary effects of copper deficiency on bone, and secondary effects due to the anemia and changes in the bone marrow. With this objective, two experiments were carried out on iron deficiency. The results are summarized in Tables I and II. In spite of the occurrence of earlier and equally severe anemia in the iron-deficient dogs, bone changes characteristic of copper deficiency were not observed as a result of iron deficiency. In some cases of iron deficiency, it appeared that the trabeculae might have been even heavier than in the controls. These observations indicated a considerable degree of specificity in the production of the bone lesions by copper deficiency.

It may be noted that iron deficiency and copper deficiency both resulted in

low levels of iron in the serum; this observation is in keeping with the hypothesis that copper-deficiency anemia is a result of defective utilization of iron.[5] However, with comparable degrees of anemia, the morphological alterations in the erythrocytic elements of the peripheral blood and bone marrow were distinctly different under the two circumstances (3), leaving doubt as to the identity of the hematological changes produced by deficiencies of the two substances, at least under the circumstances of the present experiments. The observation of normal or low levels of plasma and liver copper in the iron-deficient dogs also is somewhat at variance with results obtained in swine (11).

An incidental observation of some interest was the development of ascites and edema in the two severely anemic iron-deficient dogs. It should be noted also that, in contrast to the copper-deficient dogs, two of the iron-deficient dogs showed evidence of some retardation of epiphyseal development.

DISCUSSION

Spontaneous fractures have been observed in sheep and cattle grazing on copper-deficient pastures in various parts of the world, but the disorder has never been studied sytematically. The condition is associated with anemia, and with plasma levels of calcium, inorganic phosphorus, and alkaline phosphatase which are normal (13, 14). Teague and Carpenter (15), during the course of our own studies, reported the occurrence in copper-deficient hogs of excessively flexed hocks and crooked forelegs. No specific changes were described in the bones. In a later publication (16), it was concluded that the lameness and other alterations probably were results of anemia. Changes about the joints of hogs on modified milk diets deficient in copper have been noted also by Lahey *et al.* (11). Numerous investigations have dealt with anemia (17) and graying of the hair (18, 19) in various species as a result of copper deficiency.

The bone disorder due to copper deficiency in the present experiments developed under controlled conditions on diets of known composition. The disorder was specifically prevented, or relieved, by copper. Growth and development of the copper-deficient dogs were normal, and the bone changes were not a result of inactivity, inanition or anemia.

The effects of the calcium and phosphorus content of the diet upon the formation and structure of bone in rats have been investigated extensively by Shohl and Wolbach (20) and by Carttar, McLean and Urist (21). Perhaps

[5] The plasma iron determinations were done through the courtesy of Dr. George E. Cartwright of the Department of Internal Medicine, University of Utah, for which we are grateful.

more pertinent to the present study were the control observations on the diet employing Wesson's salt mixture by Freeman and McLean (22) in their study on the bones of growing dogs. On the basis of their calcium and phosphorus content, all of the diets of the present study fulfilled the usual criteria of "adequate" or "optimum" diets. Likewise, with the possible exception of the earliest diet, it is thought that the present diets were neither deficient, nor greatly excessive, in their content of any of the other required elements or vitamins except for copper. The results with the different diets were somewhat suggestive that the severity of the bone alterations, particularly the incidence of secondary lesions such as fractures and deformities, may have been influenced by variations, within the adequate range, of the calcium and phosphorus content of the diets. However, despite the possibility of other influencing factors, copper was the key factor throughout which determined whether or not the bone lesions developed. The control animals in all cases appeared to be normal.

The studies did not afford evidence of a primary disturbance in calcium or phosphorus metabolism as a result of copper deficiency. Plasma levels of calcium, inorganic phosphorus, and vitamin D were normal, phosphatase levels were the same as in control animals, and the changes observed roentgenographically and on inspection of the bones were not characteristic of rickets.

The nature of the bone lesions of copper deficiency will be considered in detail in the accompanying report on the histological and chemical studies on the bones. Failure of similar bone lesions to occur in iron deficiency indicated a high degree of specificity of copper deficiency in the causation of the lesions.

SUMMARY

Young dogs made severely deficient in copper developed a bone disorder characterized by abnormally thin cortices, deficient trabeculae and wide epiphyses. Fractures and deformities of the bones occurred in many of the animals. Anemia and graying of the hair accompanied the bone changes. The disorder was relieved by the administration of copper, and never occurred in control animals.

Calcium, inorganic phosphorus and vitamin D levels of the serum of deficient animals were normal, and the bone lesions were not characteristic of rickets. The bone disorder was not a result of inactivity, inanition, or anemia. The failure of iron deficiency, with severe anemia, to produce similar bone changes, indicated that the bone disorder of the present study was a specific effect of copper deficiency.

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PLATE I

FIG. 1. Photograph of three littermates from Experiment 3 after more than 3 months on purified diets. At the left is a control dog, in the center is a dog on copper-deficient diet with salt mixture No. 2, and at the right is a dog on the copper-deficient diet with the modified Wesson's mixture. The control dog was entirely normal. Hematocrits of the deficient dogs were approximately 15 per cent. Their hair was gray and rough and their legs were obviously deformed. Roentgenograms of the control dog and of the deficient dog on the right are shown respectively in Figs. 6 and 9, and in Figs. 8 and 10. Similar changes were present in the x-rays of the other deficient dog. The deficient dog at the right was larger in frame than the control, but weighed a little less.

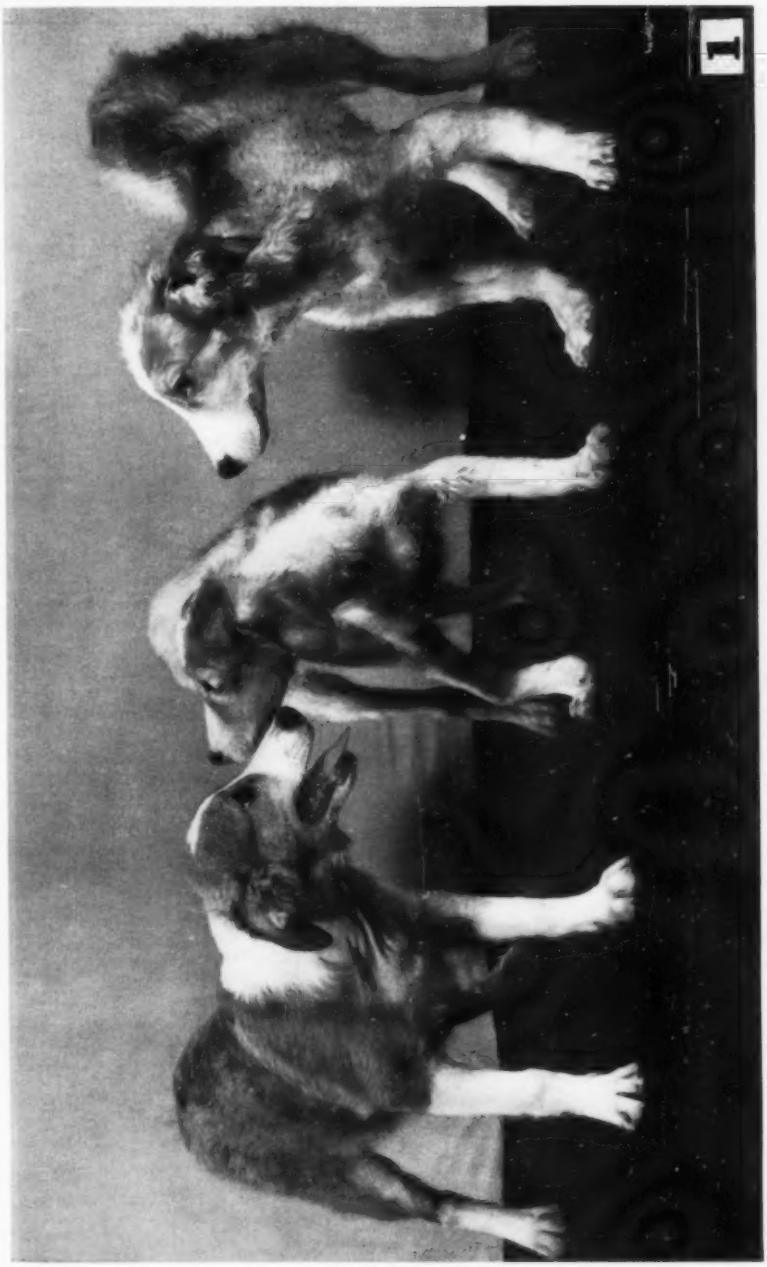
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PLATE II

FIG. 2. Side view of the copper-deficient dog on the right in Fig. 1, showing extreme degree of bowing of forelegs and roughness of hair. For x-rays of this dog see Figs. 8 and 10.

FIG. 3. Rear view of a copper-deficient dog from Experiment 7. Note the hyperextension of wrist joints and the typical knock-kneed position of the hind legs. This dog developed peculiar sacculations over the ischial tuberosity. Similar changes were observed about the joints of two other deficient animals. These sacculations contained fluid and were lined by synovial membrane.

FIG. 4. Photograph of a copper-deficient dog from Experiment 8. Deformities about the wrist joints and inward bowing of the hind legs are shown. The normally dark hair in some areas has become gray.

FIG. 5. A copper-deficient dog from Experiment 1 after treatment with copper for 1 month. This dog was unable to stand prior to treatment. Considerable improvement occurred but the hyperextension of wrists and some disturbance in the hind legs remained. In this first experiment, no x-rays were made and fractures, if present, were not recognized. These dogs were believed at the time of the experiment to be suffering from a neuromuscular disturbance. It is assumed now that the lameness was a result of bone changes.

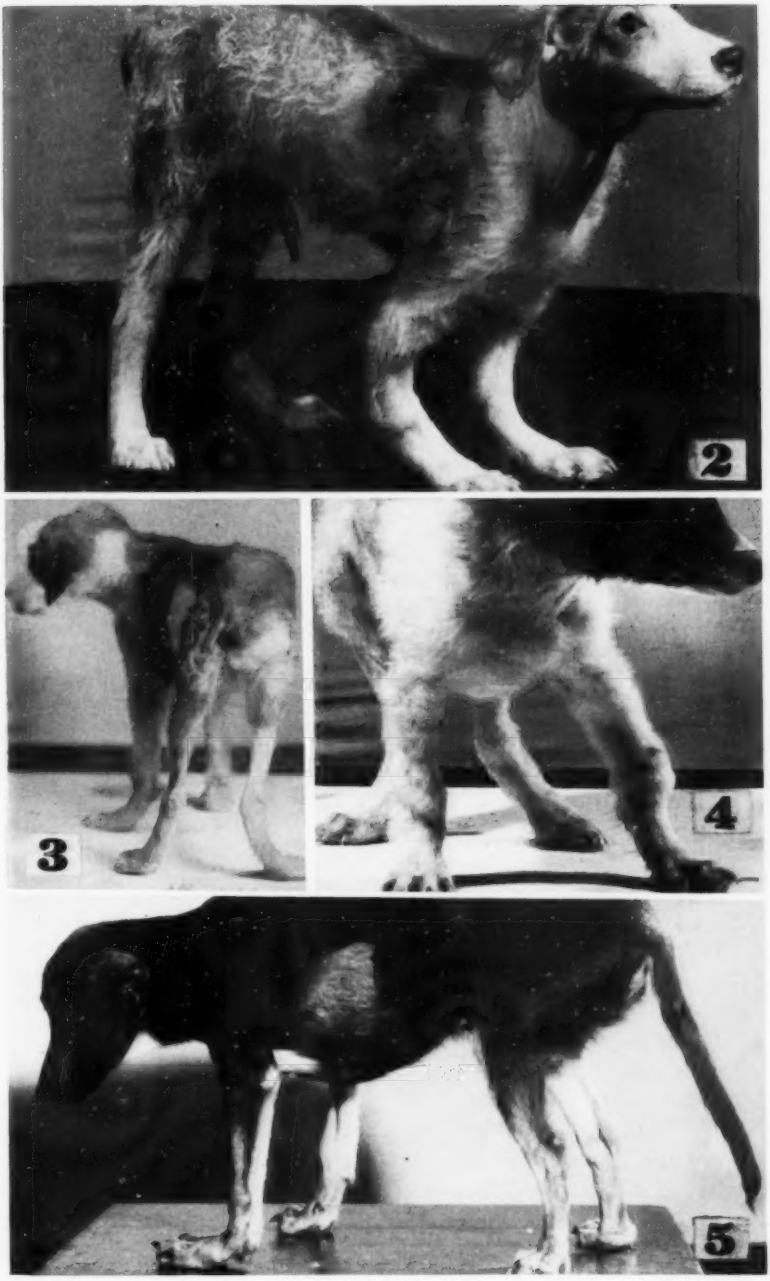
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PLATE III

All of the roentgenograms in this and the subsequent plate were made by comparable techniques and reproduced on the same scale. Because of the difficulties of reproduction, only changes of severe degree are illustrated.

FIG. 6. Roentgenogram of the foreleg of the control dog shown on the left in Fig. 1. Note the relatively thick cortices, and the prominent trabeculae in the epiphyses and small bones about the wrist.

FIG. 7. Similar roentgenogram of a copper-deficient dog. Note the thin cortices, wide medullary cavities, and absence of trabecular markings. The epiphyseal cartilages appear to be slightly wide. The metacarpals are larger than in the control dog. A fracture of the humerus occurred later in this dog.

FIG. 8. Similar roentgenogram of the deficient dog shown at right in Fig. 1. Here, in addition to the changes shown in Fig. 7, various complications have occurred. There is bowing of the radius and ulna, several fractures are present in these bones, their epiphyses are deformed, and the lower end of the radius is considerably enlarged, probably in part as a result of compression. The same changes were observed in the contralateral foreleg.

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PLATE IV

FIG. 9. Roentgenogram of a posterior extremity of the control dog of Fig. 1.

FIG. 10. Roentgenogram of the posterior extremities of the copper-deficient dog shown at right in Fig. 1. Again, note the very thin cortices, wide medullary cavities, and the "ground-glass" appearance of the bones.

FIG. 11. Fracture of the humerus in a copper-deficient dog. This fracture occurred from slight stress while restraining the dog for venipuncture.

FIG. 12. Fracture of the femur of the same dog. This dog improved dramatically when treated with copper and the appearance of the bones in x-rays became normal.

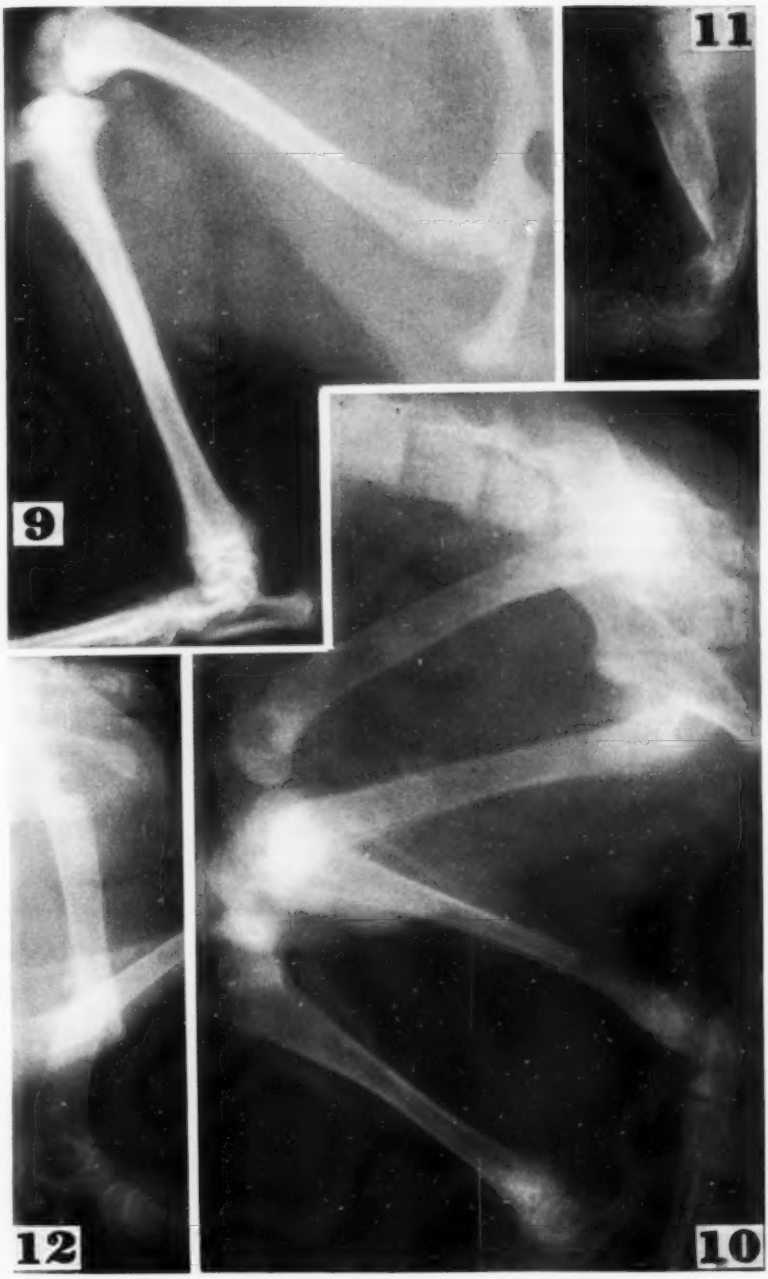
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PLATE V

FIG. 13. In this and the subsequent figure, a bone from a control dog is shown at the left and the corresponding bone from a copper-deficient dog at the right. In contrast to the roentgenograms which illustrate severe bone changes, these photographs illustrate the milder changes which occurred.

Third metacarpals are shown in this figure. Note that the bone from the deficient animal was larger, with thinner cortices and a wider medullary cavity. ×1.4.

FIG. 14. Lower ends of tibiae illustrate the same differences. In addition, it can be seen that the bone of the deficient animal had more delicate trabeculae, less blood in the peripheral portions of the cortices, and a wider epiphyseal cartilage. ×1.5.

13

14

A BONE DISORDER ASSOCIATED WITH COPPER DEFICIENCY

II. Histological and Chemical Studies on the Bones

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In the preceding report (1), a description was presented of the gross morphological, roentgenological and chemical observations on young dogs made severely deficient in copper. The major attention was devoted to the bone alterations. In this second report of the series, further observations on the bone disorder are described with particular attention to the histological and chemical characteristics of the bones of the copper deficient animals.

METHODS

The animals, diets and general plan of the experiments have been described (1).

The proximal ends and diaphyses of the femurs, distal ends of second metacarpals, and costochondral junctions of fifth or sixth ribs from the autopsied animals of each experiment were fixed in 10 per cent neutral formalin, decalcified, imbedded in paraffin or nitrocellulose, sectioned, and stained with hematoxylin and eosin or Giemsa's stain.

Bones were prepared for chemical determinations by stripping of periosteum, splitting longitudinally with a scroll saw and washing out the marrow in a jet of water (2). The determinations were carried out on cortical bone prepared by cutting away the ends, in the case of limb bone, and by curetting out the trabeculae. The bones were stored in 95% neutral ethanol until ready for use.

Samples for ashing were extracted for 20 hours in a Soxhlet extractor with methanol distilled from calcium oxide, and dried at 105°C. The dry, fat-free cortical bone was weighed into crucibles and ashed to constant weight at 500–550°C in an electric muffle furnace.

The other analyses were performed on cortical bone powder prepared by the method of Bogert and Hastings (3). The carbonate determinations were made in triplicate in a Van Slyke-Neill manometric apparatus equipped with a separate combustion chamber according to the method of Danielson and Hastings (4). The calcium and phosphorus determinations were carried out on deproteinized solutions of the bone powder. Calcium was determined by the Clark-Collip procedure (5), and phosphorus by the Fiske-SubbaRow method (6). Calculations were made of the residual calcium to phosphorus ratio, and of the calcium phosphate to calcium carbonate ratio.

RESULTS

Histological Studies

Microscopic examinations of the bones of the copper-deficient animals served to confirm the gross impressions of cortical and metaphyseal thinning and increase in width of epiphyseal and costal cartilages (1).

[1] Public Health Service, Department of Health, Education and Welfare.

When the cortices of the copper-deficient animals were compared with those of the controls (Figs. 1–6) several important differences were readily apparent. In the first place, the bones from the deficient animals in severe cases were only one-third to one-half as thick as those of the controls. Within the depth of cortical bone there was no particular difference in vascularity, and the blood vessels in the Haversian canals in the centers of individual osteones were approximately the same size. The osteones themselves did not appear to differ insofar as the distribution of their concentric rings of osteocytes were concerned. Obviously there were fewer osteones in the bones of the copper-deficient animals. In the subperiosteal regions there was definite evidence of increased cortical resorption as well as cellular proliferation in the deficient group. There were spaces within the bone containing blood vessels, osteoblasts, and occasional osteoclasts. Most of the external surfaces of the cortices of deficient animals had irregular or "saw tooth" margins as a result of lacunar absorption. In such lacunae osteoclasts usually were found.

An even more striking difference between deficient animals and their controls was found in the inner portion of the cortex bounding the medullary cavity (Figs. 3 and 4). Here there was even greater evidence of excessive absorption or destruction. The inner layer of what in the controls was compact osseous tissue had been converted into a sort of cancellous osseous tissue. One found, therefore, thin lamellae or trabeculae which appeared to have been formed by erosion of the overlying compact cortex. The intercancellous spaces contained blood vessels, osteoblasts, a few osteoclasts and connective tissue cells. The latter elements were particularly prominent in juxta-position between the inner margin of cancellous bone and marrow cells. The erythroid and myeloid components of the marrow nowhere appeared to be encroaching directly on the bone. In certain experiments, bones from both copper-deficient and control animals were observed to have osteoid borders along certain of the trabeculae of the inner cancellous portions of the cortex.

The shaftward portions of the metaphysis showed changes similar to those of the inner portions of the cortex bounding the medullary cavity. The trabeculae were thinner than normal, more widely spaced, less definitely oriented in a longitudinal direction, and they showed evidence of erosion and/or lack of bone deposition. The same changes were present in the centers of ossification of the epiphyses.

The picture in the cortex, therefore, was one of excessive absorption so that the normal balance of periosteal deposition versus central absorption was shifted toward a predominance of the latter phenomenon. Trabeculae of the shaftward portion of the metaphysis showed similar changes.

When one examined and compared the epiphyseal cartilages and the portions of the metaphyses adjacent to the epiphyses, a most unusual picture

was found. The epiphyseal cartilage was wider in the deficient animals (Figs. 7, 8, 9, 10, 13, and 14). This was the result of a definite difference in the proliferative activity of the cartilage cells. The zones of proliferating cells and of mature hypertrophic cells were wider in the deficient dogs than in the controls. The junction between the cartilage cells and the metaphysis was regular. There was no irregularity in invasion by blood vessels. Neither was there any evidence of defective deposition of inorganic materials in the cartilage matrix between the hypertrophic cells.

In the juxta-epiphyseal metaphysis the spicules of calcified matrix ("lattice") were thinner, though often more numerous, than those of the controls (Figs. 11, 12, 15, and 16). In addition, there was more than normal cross-bridging between the spicules. These changes gave the juxta-epiphyseal metaphysis of the deficient animals a denser, more disorderly appearance.

In summary then, at the cartilage shaft junction there appeared to be an over-activity or perhaps over-prolongation of activity of the epiphyseal and costal cartilage, together with a persistence of the lattice of calcified cartilaginous matrix and a reduction of bone formation on this structure.

It has already been noted (1) that iron-deficient dogs, even with severe anemia, failed to develop the bone changes in the gross which were associated with copper deficiency. Likewise, microscopic examination of comparable bones from the iron-deficient dogs failed to reveal the alterations which have been described here in association with copper deficiency.

The bone marrow of copper-deficient animals differed in appearance and staining characteristcs from that of the controls and also from that of iron-deficient dogs. The marrow is described in detail in the following report (7). It has already been pointed out that erosion of the cortices in the copper-deficient dogs did not appear to be a result of direct impingement on the bone by the erythrocytic and granulocytic cells of the marrow. Further evidence against "mechanical" effects on the bone by hyperplastic marrow in copper deficient animals was afforded by the lack of correlation in certain areas, particularly in the metacarpals, between cellularity of the marrow and thinning of adjacent bone trabeculae.

Chemical Studies

The results of determinations of ash, calcium, phosphorus and carbon dioxide on cortical bone from control and copper-deficient animals are shown in Table I. The ratios of residual calcium to phosphorus and of calcium phosphate to calcium carbonate also are indicated. The results were almost identical in the two groups of animals.

TABLE I

Percentage Composition of Dry, Fat-Free, Cortical Bone

	NORMAL CONTROL DOGS*	COPPER-DEFICIENT DOGS*
Ash	63.5 (61.4–64.4)	63.7 (61.8–65.6)
Calcium	24.3 (23.9–24.6)	24.8 (24.7–25.5)
Phosphorus	11.2 (11.0–11.6)	11.5 (10.8–12.3)
Carbon dioxide	2.58 (2.41–2.71)	2.65 (2.49–2.79)
Residual Ca:P, Wt. ratio	1 97 (1.94–2.01)	1.96 (1 88–2 10)
$Ca_3(PO_4)_2$:$CaCO_3$, Wt. ratio	9.6 (8.9–10.5)	9.6 (8.9–10.4)

* The averages of 7 ash determinations and 6 determinations of Ca, P and CO_2 in each group are shown, with the range in parenthesis. Bones from animals of experiments 7 and 8 were used.

The results of Ca determinations were corrected on the basis of calcium standards which were run simultaneously through the procedure with a systematic loss of approximately 7 per cent.

DISCUSSION

The skeletal changes we have observed in these dogs are not unexpected since bone changes have been described in association with copper deficiency under both experimental and natural conditions. Studies of experimental copper deficiency in swine by Teague and Carpenter (8) and Lahey *et al.* (9) have revealed deformities of the extremities. Roentgenographic and morphologic examination of such limb bones have not been reported. Both Davis (10) and Cunningham (11) have described gross skeletal defects associated with copper deficiency in cattle and lambs. Davis refers to the bone fractures and enlargement of the joints as a "condition of osteomalacia". This is an unfortunate and misleading designation since the diagnosis of *osteomalacia*, i.e., defective deposition of inorganic materials in bone matrix, can only be made by chemical or histological examination of the tissue. Doubtless, Davis used the term "osteomalacia" in the sense of bone rarefaction for which the term *osteoporosis* should be used.

Cunningham (10) stated that in the bones in which fractures had occurred there were no macroscopic changes to account for the breaks. In common laboratory animals, i.e., rats and rabbits, bone changes have not been specifically noted when such species were placed on a copper deficient regimen. Changes in the skeletal tissues of dogs have not been heretofore reported.

In any discussion of the pathogenesis of bone disease it is necessary to bear in mind several important aspects of normal skeletal growth because abnormalities can best be understood in terms of the normal. Three mechanisms must be considered as far as normal bone formation is concerned: growth of cartilage, balance of osteogenic and osteolytic activities, and the deposition of

inorganic materials in the organic matrix of cartilage and bone. It must be further emphasized that the activities of cartilage cells and osteoblasts are not necessarily mutually interrelated; that is, there may be complete dissociation of the functional activity of these two elements.

The microscopic changes which have been described above in the copper-deficient dogs exemplified certain perversions of the normal pattern. It may be said at the outset that their pathogenesis can only be surmised.

The alterations in the diaphyses (cortices) and the shaftward portions of the metaphyses have been interpreted as resulting from excessive destructive activity. This increased destruction apparently had been very gradual so that one would not have expected it to be manifested by changes in the serum concentrations of calcium or phosphorus. Whether metabolic evidence of excess destruction could be derived from calcium and phosphorus balance studies remains an open question. In addition to the increased destruction, there may well have been decreased osteoblastic activity in these regions. The evidence for this is mainly inferential, based on the decreased osteoblastic activity in the juxta-epiphyseal metaphysis, i.e., lack of bone matrix deposition on the calcified cartilaginous "lattice". It may be that copper is necessary for normal osteoblastic function just as are other essential nutrients, of which the example *par excellence* is ascorbic acid.

When we came to consider the epiphyseal or costal cartilages the picture was more complex. Here over-proliferation of cartilage seemed to be occurring. Deposition of inorganic elements was not abnormal. Osteoblastic activity, both constructive and destructive, was impaired as evidenced by a persistence of the calcified cartilage lattice covered by a diminished amount of bone. Generally, when osteoblastic activity is depressed, as in inanition, growth of the cartilage is also affected. This is not invariable, however; for instance, in ascorbic acid deficiency, although osteoblastic activity is greatly affected, the proliferative potential of the cartilage is not affected. The same dissociation is seen in the osteogenesis imperfecta syndrome.

The possibility that hyperplasia of the marrow elements might have played a role in the thinning of the bones must be considered. As noted above, there appeared to be no encroachment by these cells on the bone. Moreover, in iron deficient animals with hyperplastic marrow, no bone changes appeared.

One can only suggest that copper deficiency may lead to derangement in the metabolism of the osteoblast while the cartilage cell is quite immune to a lack of this essential nutrient. It is hazardous to speculate too far; however, it is of interest to point out that we have likened the picture observed in these copper-deficient dogs to what one might see in ascorbic acid deficiency, i.e., a derangement in osteoblastic activity as far as the elaboration of bone matrix is concerned together with a cessation of destruction of the calcified cartilaginous matrix while chondroblastic activity is not specifically impaired. The

possible role of copper in the metabolism of ascorbic acid and the aromatic amino acids is now being investigated. It is of interest to note that we have also observed a derangement of maturation of the cells of the erythrocytic series (7) in dogs as a result of deficiency of copper.

The chemical studies on cortical bone seemed adequate to rule out any serious abnormality of mineralization in the control dogs and to indicate that the bone disorder in the copper-deficient dogs was not a result of defective mineralization. The absence of chemical changes in the inorganic substance of the bone of the copper-deficient animals is in line with the observation of normal levels of calcium, phosphorus, and vitamin D in the blood (1). All of the morphological and chemical observations support the view that copper deficiency was associated with a diffuse osteoporosis of the skeleton, without any primary abnormality in the calcification mechanism.

SUMMARY

Microscopic studies indicated that in the copper-deficient animals there was excessive absorption of bone, particularly about the inner portions of the cortex and also in the shaftward portions of the metaphyses. In addition to increased destruction, decreased deposition of bone matrix may have occurred. These changes did not appear to be related to the bone marrow hyperplasia.

At the cartilage-shaft junctions, there appeared, on one hand, to be overproliferation of the cartilage cells, while on the metaphyseal side, there was abnormal persistence of the lattice of calcified cartilaginous matrix with a reduction in deposition of bone matrix on this structure.

Chemical studies on cortical bone showed that ash, calcium, phosphorus, and carbon dioxide were normal in controls and copper-deficient dogs. The residual Ca:P ratio and ratio of $Ca_3(PO_4)_3$:$CaCO_3$ were almost identical in the two groups.

It was concluded that the bone disorder consisted of a diffuse osteoporosis, with no primary disturbance in the calcification mechanism. Possible mechanisms involved in the bone disorder are discussed briefly.

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PLATE I

FIG. 1. Low power photomicrograph of mid diaphysis of femur of control dog (No. 7-4).

FIG. 2. Low power photomicrograph of same region copper deficient animal (No. 7-2). Note difference in width as compared with Fig. 1. Note cancellous transformation of inner surface and lack of marrow encroachment on this surface.

FIG. 3. Higher power of inner cortex shown in Fig. 1. Note normal vascularity of this region. Thin osteoid borders are present.

FIG. 4. Higher power of inner cortex shown in Fig. 2. Note more vascularity than that found in Fig. 3. Same amount of osteoid appears to be present. Note also loose connective tissue layer between marrow cells and endosteum.

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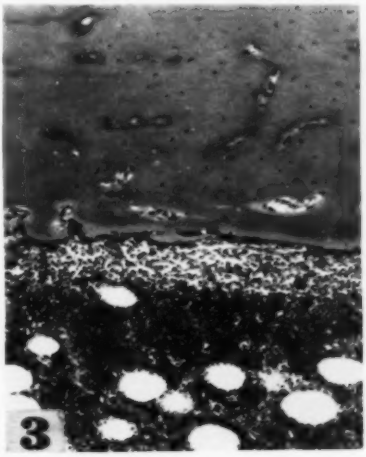
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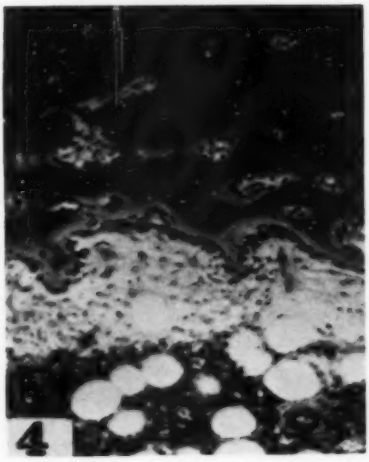
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PLATE II

FIG. 5. Low power photomicrograph of cortex of control dog (No. 8-1).

FIG. 6. Low power photomicrograph of cortex from copper deficient animal (No. 8-6) to compare with Fig. 5.

FIG. 7. Epiphyseal cartilage and metaphysis of rib of control dog.

FIG. 8. Epiphyseal cartilage and metaphysis of copper deficient dog. Note increase in width of proliferating cell zone. Also note absence of irregularity in invasion and increase in cartilage matrix beneath cartilage cells so that this area appears denser than controls.

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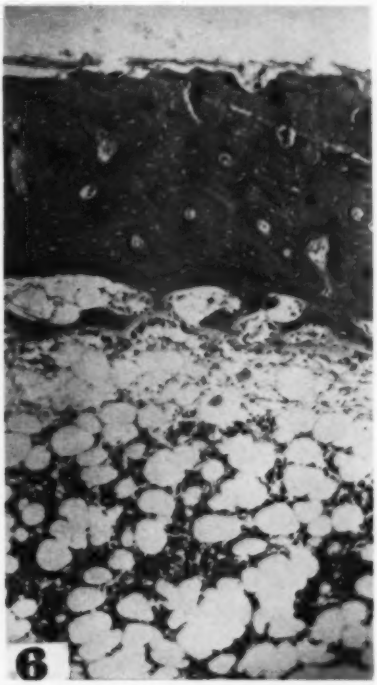
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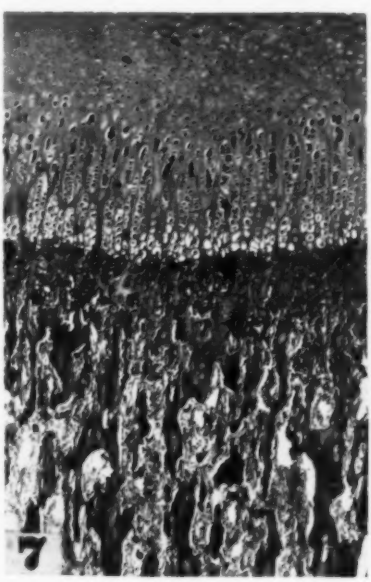
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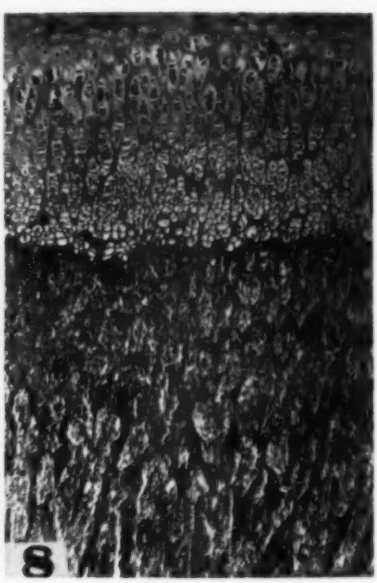
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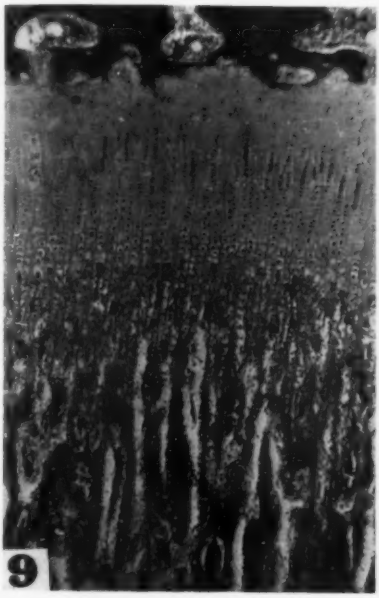
PLATE III

FIG. 9. Cartilage shaft junction of upper femur of control dog (No. 7-4).

FIG. 10. Cartilage shaft junction of upper femur of copper deficient dog (No. 7-2). Note increase in width and some increase in density of subcartilaginous area.

FIG. 11. Higher power of junction between cartilage and shaft shown in Fig. 9.

FIG. 12. High power of junction between cartilage and shaft shown in Fig. 10.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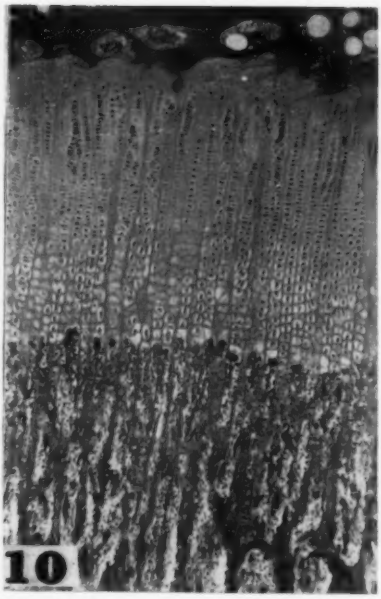
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PLATE IV

FIG. 13. Cartilage shaft junction of upper femur of normal dog (No. 8-1).

FIG. 14. Cartilage shaft junction of upper femur of copper deficient dog (No. 8-6). Note increase in width of cartilage and dense zone of calcified cartilaginous matrix in upper metaphysis.

FIG. 15. Higher magnification of region of upper metaphysis of Fig. 13.

FIG. 16. Higher magnification of region of upper metaphysis shown in Fig. 14.

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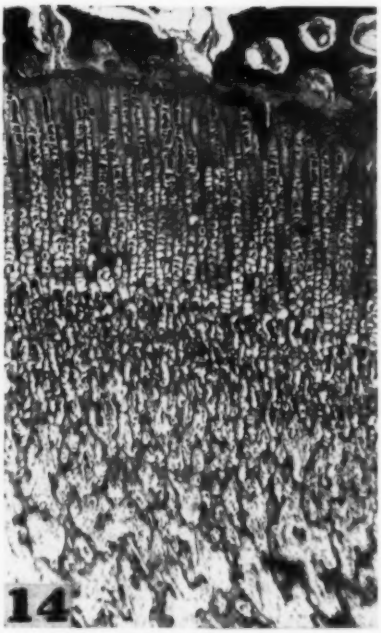

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THE ANEMIA OF COPPER DEFICIENCY IN DOGS COMPARED WITH THAT PRODUCED BY IRON DEFICIENCY

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The occurrence of a bone disorder in young dogs maintained for several months on diets greatly deficient in copper, has been described in the preceding reports (1, 2). In order to determine whether the bone changes were specific effects of copper deficiency, or results of anemia and bone marrow hyperplasia, observations were carried out on similar dogs maintained on diets containing adequate amounts of copper but inadequate amounts of iron. It has already been noted (1) that the iron-deficient dogs, despite the development of severe anemia, failed to develop bone alterations of the type which occurred in copper deficiency.

On the basis of observations of other investigators employing swine (3), the present experiments with iron deficiency in dogs were undertaken with the assumption that hematological changes identical with those resulting from copper deficiency would be produced. However, it soon became apparent that, with comparable degrees of anemia, the changes in red cell size and hemoglobin content were much greater in iron deficiency than in copper deficiency. Likewise, the bone marrow changes differed in certain respects under the two circumstances. From the present observations in copper deficiency, it did not seem likely that the hematological alterations could be explained on the basis of a deficient formation of hemoglobin; rather, there appeared to be a deficient production of mature red blood cells.

METHODS

The animal experiments in which the present observations were made have been described adequately (1). Dogs were maintained on the experimental diets for 100 to 150 days. Nineteen control dogs, 27 copper-deficient dogs, and 5 iron-deficient dogs were studied. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoblogin concentration (MCHC) were determined by the methods of Wintrobe (4), near the end of each experiment and in some cases several times during the course of the experiments. Hemoglobin was determined colorimetrically by the method of Evelyn (5). Counts of white cells, reticulocytes, and platelets, and studies of smears of peripheral blood were carried out in some experiments.

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In experiment 13 (1), serial observations were made on bone marrow smears from control, copper-deficient, and iron-deficient dogs. The bone marrow was aspirated from the spinous processes using a University of Illinois sternal needle. In other experiments, post-mortem bone marrow smears and sections were studied.

RESULTS

Peripheral Blood. The peripheral blood picture of the iron-deficient dogs was the classical one which has been described in dogs and other species. The red cells appeared small and pale, particularly in the centers, with moderate anisocytosis, poikilocytosis and polychromatophilia (Figs. 1, 2). The total red cell counts remained near normal, or even above normal, in the face of severe anemia as indicated by hemoglobin determinations. There was a marked increase in platelets, which varied in shape and in size from normal to several times normal. The white blood cells appeared to be normal quantitatively and qualitatively.

In contrast, the peripheral blood of the copper-deficient dogs appeared almost normal morphologically (Fig. 3). The red cells were normochromic, with minimal anisocytosis and poikilocytosis. Reticulocytes were decreased. There was a moderate increase in platelets, which were large and of fairly uniform size. The granules of the platelets in blood films from copper-deficient dogs were larger and stained more intensely than those from normal or iron-deficient dogs. White cell counts in the copper-deficient dogs were normal or slightly decreased.

Spherocytes were not noted in blood smears from copper-deficient dogs. Icterus of mucous membranes was never observed and the plasma of anemic dogs uniformly appeared to contain normal amounts of bile pigment. It has already been noted that approximately normal quantities of hemosiderin were noted in the spleen, liver, and bone marrow (1). From these observations it appeared unlikely that hemolysis played a role in the anemia due to copper deficiency. A mild decrease in survival time of the red cells was not ruled out.

The hematological indices of control, iron-deficient, and copper-deficient animals plotted against the degree of anemia as measured by the hemoglobin content of whole blood are shown in Chart 1. The control values are similar to those observed by Wintrobe *et al.* (6). In the iron-deficient dogs, the MCV, MCH, and MCHC fell progressively as the degree of anemia increased. In contrast, the indices in the copper-deficient dogs remained normal even with extreme degrees of anemia. Indices were still normal after a significant degree of anemia had been present for 1 to 2 months.

Bone Marrow.—Marrow from the iron-deficient dogs appeared moderately hyperplastic, due to an increase in normoblasts. The ratio of cells of the granulocytic series to nucleated cells of the erythrocytic series was reduced

in severely anemic animals to approximately 0.8, compared with a control value of 1.5 or greater. Granulopoiesis did not appear to be disturbed. Thrombopoiesis was accelerated as evidenced by irregular platelet masses about intact

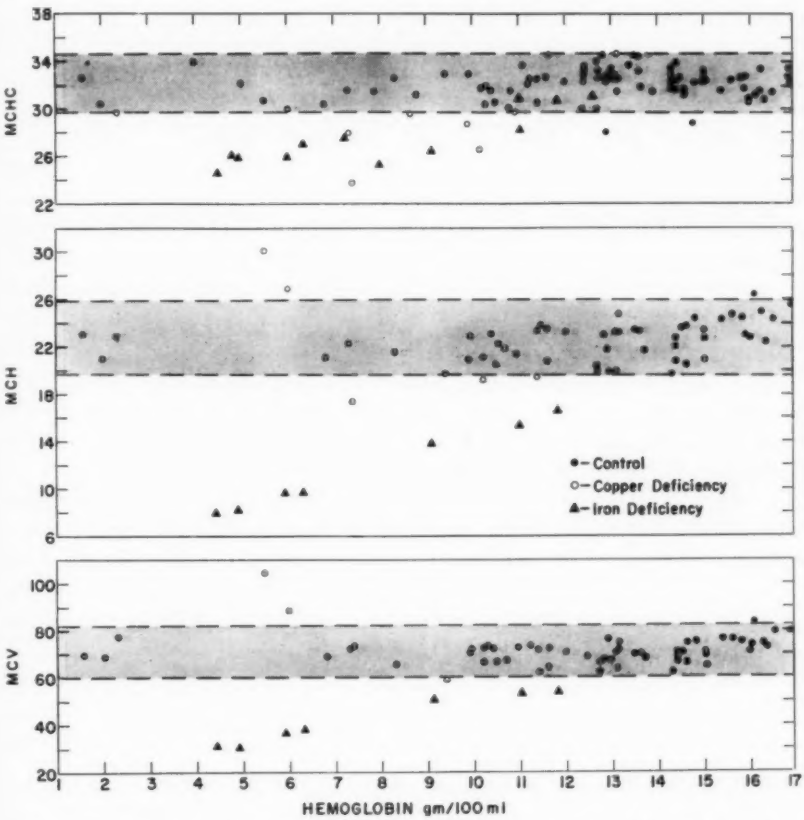

CHART 1. Hematological indices of control, copper-deficient, and iron-deficient dogs plotted against degree of anemia as measured by hemoglobin determinations. The shaded areas have no significance except to indicate the range within which most of the control values fell. All determinations are included. All of the dogs described in the previous report are represented in the chart. Complete indices were determined at least once in each experiment except Exp. 12.

megacaryocytic nuclei. Of the increased number of normoblasts, the majority were in the later stages of development as indicated by their size and nuclear characteristics. These cells contained nuclei of approximately normal size, but the cytoplasm was reduced to narrow rims and the hemoglobin content

was decidedly subnormal. The number of mitotic figures in the developing red cells did not appear to be increased.

In the copper-deficient dogs the marrow appeared to be more hyperplastic than that of the iron-deficient animals. The average ratio of granulocytic cells to nucleated erythrocytic cells in severely anemic animals was 0.4. The distribution of cells of the erythrocytic series was shifted decidedly to the left, with a decrease in the proportion of late normoblasts and an increase in the number of more immature forms with larger nuclei and more basophilic cytoplasm (Fig. 4). There were evidences of some dissociation between cytoplasmic and nuclear maturation. Even normoblasts with orthochromatic cytoplasm in some cases contained nuclei which appeared large and immature, and small normoblasts with pyknotic nuclei sometimes were observed to have prominent basophilic material in the cytoplasm. Cellular hyperactivity was evidenced by the increase in number of mitotic figures seen in all stages of developing red cells. These observations were indicative of a defective maturation of the erythrocytic elements. The quantity of cytoplasm and the hemoglobin content of the late normoblasts appeared comparable to those of control animals, and greater than those of iron-deficient animals. Granulopoiesis did not appear to be disturbed significantly. Megacaryocytes and platelets were increased, but not to the extent seen in iron deficiency.

An interesting observation was the appearance of large numbers of osteoblasts and fewer osteoclasts in smears of bone marrow aspirated from two of the copper-deficient dogs (Figs. 5 and 6). It seemed likely that the bone marrow in these cases was aspirated from areas adjacent to the inner surface of the bone cortex, and that the cells were the same that have been described about the eroded endosteal bone surfaces in the preceding report (2). Small numbers of similar cells were observed in smears from control and iron-deficient animals.

DISCUSSION

There is little disagreement among various observers concerning the changes which occur in the peripheral blood of various species as a result of deficiency of iron. However, in copper deficiency the morphological changes described by different observers and in different species have varied rather widely. These observations have been summarized recently by Lahey *et al.* (3). The present observation of essentially normal indices in dogs made anemic as a result of copper deficiency are in agreement with the findings of Maass and coworkers (7).

Since anemia developed earlier in the iron-deficient dogs than in the copper-deficient dogs, the question arises as to whether the normochromic normocytic cells of the copper-deficient animals might not have developed before the occurrence of severe copper deficiency. This appears unlikely since normal red cell indices were observed in dogs which had been anemic, as a result of

copper deficiency, for periods as long as 2 months. Furthermore, the observation in the marrow of copper-deficient animals of normoblasts with adequate quantities of cytoplasm and hemoglobin suggested that the erythrocytes currently entering the circulation even after the development of severe anemia were still normal in size and hemoglobin content.

Lahey *et al.* (3) observed almost identical changes in hematological indices in swine as a result of iron deficiency and copper deficiency, though the leukocyte:erythroid ratio in the bone marrow under the two circumstances differed in the same direction as seen in our dogs; the similarity of the hematological changes in the swine was interpreted as indicating that a common mechanism (i.e. lack of available iron) was involved in the production of the anemia in both types of deficiency. Studies on iron metabolism in copper deficiency appeared to support this hypothesis (8). However, the observations on peripheral blood in copper deficiency in the present experiments seemed to indicate that the anemia was a result of a diminished production of red cells themselves rather than a decreased production of hemoglobin. This conclusion is given some support by previous observations in dogs (7), and by the observation that rats (9) with hypochromic, microcytic anemia as a result of combined iron and copper deficiency respond to copper by an increase in number of red cells in the peripheral blood without an increase in hemoglobin concentration. Furthermore, the present observations indicative of disorderly maturation of the erythrocytic series in bone marrow in the copper-deficient animals suggested that copper may play a role in the maturation of erythrocytes distinct from any function it may have in hemoglobin formation. Granting that copper may be involved in the absorption, mobilization, and utilization of iron in hemoglobin formation, and that copper-deficiency may under certain circumstances cause anemia as a result of hemoglobin deficiency, we believe that the evidence is not conclusive that copper is involved in erythropoiesis only as it may affect hemoglobin synthesis. In the present experiments, the anemia due to copper deficiency appeared to be the result of a defective production of red cells.

SUMMARY

Dogs made anemic as a result of iron deficiency were observed to have the classical changes in peripheral blood consisting of relatively normal numbers of red blood cells, with a marked decrease in red cell size, hemoglobin content and hemoglobin concentration. In the bone marrow, iron deficiency resulted in hyperplasia of hemoglobin-deficient normoblasts, with no appreciable shift to the left or evidence of a disorder of maturation. This is in line with the generally accepted conclusion that the hemopoietic abnormality of iron deficiency can be explained solely on the basis of a deficiency of hemoglobin.

In contrast to the anemia of iron deficiency, the anemia which occurred as a result of copper deficiency was characterized by a reduction in the number

of erythrocytes, with the maintenance of relatively normal red cell indices. In the marrow, there was no evidence of a deficiency in hemoglobin content but rather evidence of defective development of cells of the erythrocytic series.

Irrespective of any role that copper may play in the metabolism of iron and the formation of hemoglobin, the present observations were suggestive that, at least in the dog, copper is essential for the normal maturation of the erythrocytic elements of the marrow and the production of normal numbers of red blood cells.

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PLATE I

Fig. 1. Peripheral blood smear from control dog without anemia.

Fig. 2. Similar smear from iron-deficient dog with hemoglobin level of about 5 gm. Note that the red cells were small and pale. Platelets were increased in size and in number.

Fig. 3. Comparable smear from copper-deficient dog with hemoglobin level of approximately 2 gm. The red cells were similar in appearance to those of the control dog shown in Fig. 1. Platelets were large and prominent.

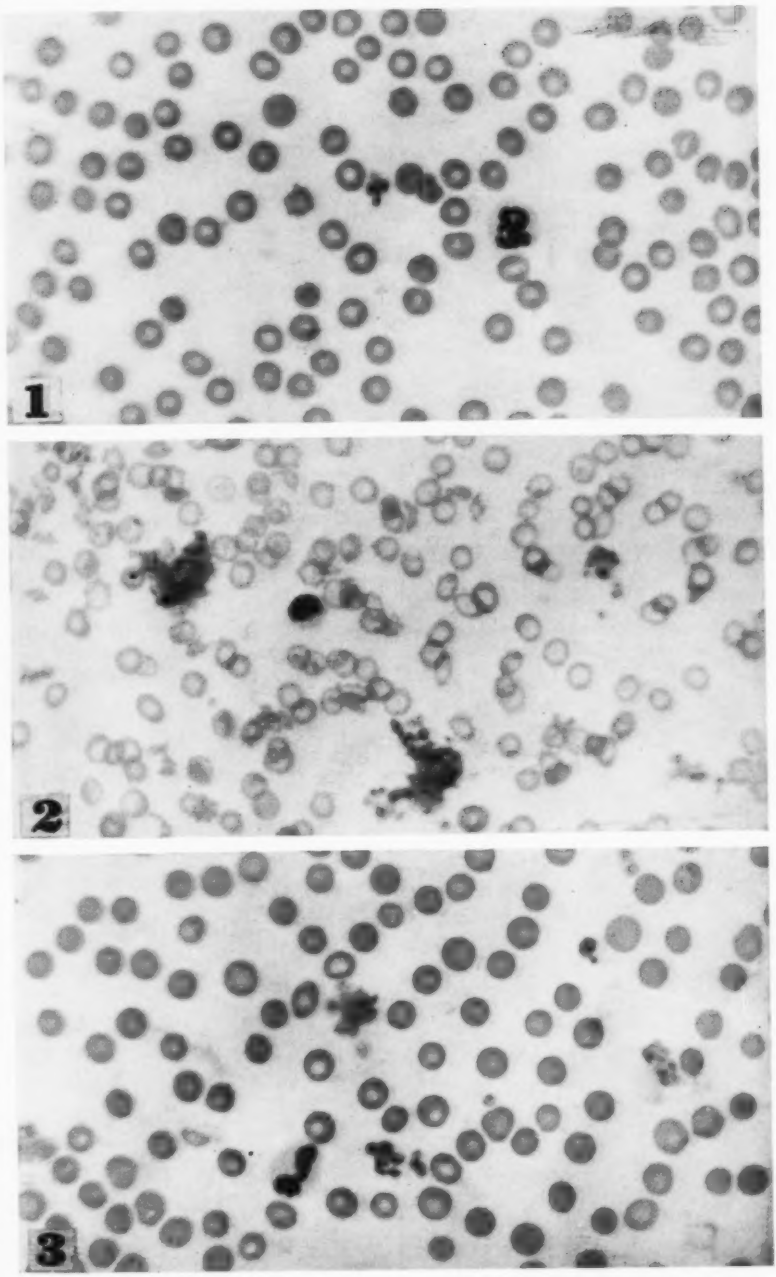
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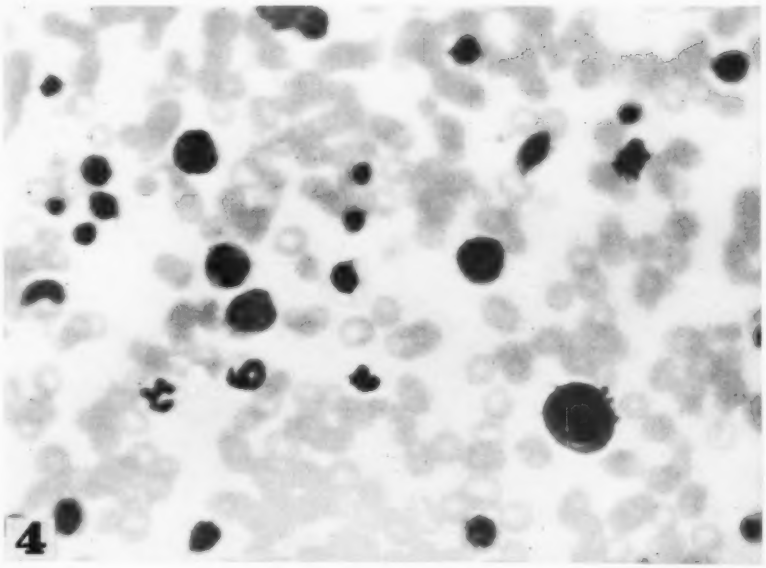
PLATE II

FIG. 4. Smear of marrow aspirated from spinous process of copper-deficient dog. The increase in proportion of developing red cells of various stages is apparent. At the bottom, to right of center, is an orthochromatic normoblast with large nucleus. Above this cell and to the right is a very large basophilic normoblast. On either side of the center of the field are similar polychromatic normoblasts with large nuclei, and above the one at the left is a large orthochromatic normoblast in mitosis.

FIG. 5. An osteoclast in a similar smear from a copper-deficient dog.

FIG. 6. A group of osteoblasts or endosteal cells in a marrow smear from a copper-deficient dog.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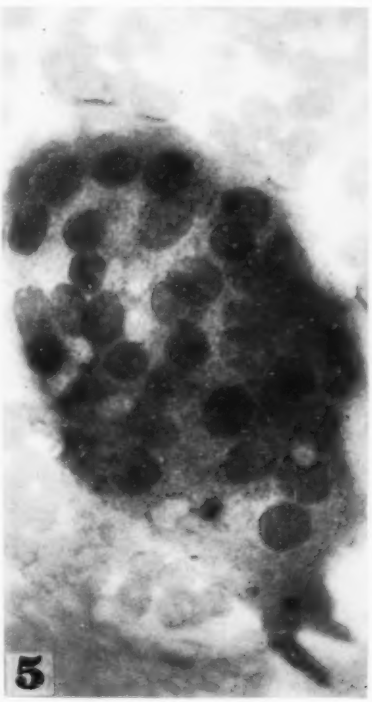
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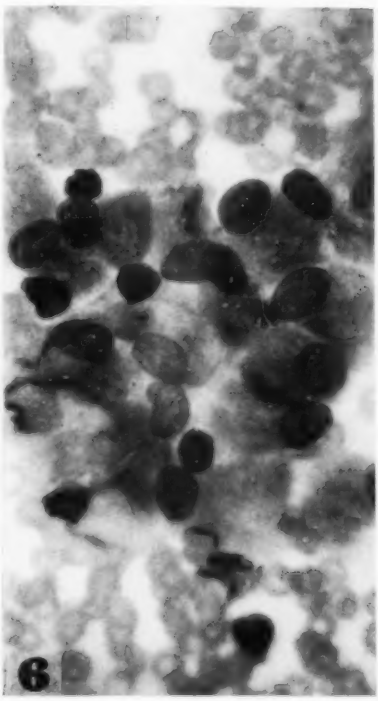
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THE FILTER PAPER ELECTROPHORETIC IDENTIFICATION OF URINARY CHORIONIC GONADOTROPHIN

A New Test for Pregnancy

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Received for publication June 3, 1953

Chorionic gonadotrophin, the pregnancy urine hormone, is a glycoprotein which is excreted, especially during early pregnancy, in easily detectable amounts. As the protein content of the urine of normal non-pregnant women is exceedingly low, it therefore seemed feasible to obtain a filter paper electrophoretic pattern characteristic of chorionic gonadotrophin from human pregnancy urine (1). It was hoped that the procedure might prove clinically valuable as a non-biological test for pregnancy.

To date samples of urine from 20 pregnant women and 12 non-pregnant women have been examined. Purified chorionic gonadotrophin has been added to both pregnancy urine samples and control samples. In addition the urine of a patient with proteinuria has been examined as well as urine from two women with ovarian failure and high urinary pituitary gonadotrophic titres.

METHODS

The protein fractions were prepared from urine samples using a modification of the Scott technique (2) for chorionic gonadotrophin assay. Fifty milliliters of a concentrated first morning urine specimen (sp. gr. 1.015 or above) were diluted to 100 ml. with distilled water and adjusted to pH 4 with 20% hydrochloric acid. The protein was adsorbed on 5 ml. of a 20% aqueous suspension of acid washed kaolin by thorough mixing. After settling, the supernatant urine was decanted and the kaolin eluted with 5 ml. of 0.1 N sodium hydroxide and centrifuged. The supernatant fluid containing the active fraction, was decanted and the kaolin discarded. For additional purification the pH of this solution was adjusted to 4.5 with glacial acetic acid and the protein precipitated by the addition of 8 volumes of 95% alcohol. The precipitate was collected by centrifugation and dried in vacuum. It was then suitable for filter paper electrophoresis. A direct alcohol precipitation of urine was tried but proved to be completely unsatisfactory.

The following conditions were maintained during the electrophoretic determinations; potential gradient 20 v./cm.; pH 8.6; molarity 0.005; buffer, barbital; temperature 20°C.; enclosed, water saturated, air atmosphere. The dried urinary alcohol precipitates were suspended in 0.1 ml. of the barbital buffer (0.005 M, pH 8.6) and a constant volume of 0.02 ml. of the suspension was added to the Whatman 3 MM filter paper strips. Migration was allowed for two hours.

The fixation and identification of the urinary protein on the filter paper strips was accomplished by routine techniques. The strips were dried at 100°C., fixed by soaking in

Supported in part by a grant from the Planned Parenthood of America., Inc., Robert L. Dickinson Memorial.

absolute alcohol saturated with $HgCl_2$, and stained with a 0.1% solution of Bromphenol blue in 1% acetic acid saturated with $HgCl_2$. The excess dye was removed by washing with 1% acetic acid and water and, after partial drying, the color was intensified by exposure to ammonia vapors.

RESULTS

Using the method described the urine of normal, non-pregnant women and pregnant women was examined, before and after the addition of a purified preparation of chorionic gonadotrophin.*

The 1st morning urine specimens from 20 women known to be in the first trimester of pregnancy, were examined. Nineteen of these contained a protein which migrated toward the cathode in a uniform manner irrespective of concentration. In one specimen it was impossible to demonstrate such a protein.

The 1st morning urine specimens from 12 women known not to be pregnant were examined under similar conditions. In no case was any protein found to migrate toward the cathode.

When purified chorionic gonadotrophin was added to the non-pregnant urine samples, a spot appeared on the filter paper in the region of the cathode. This spot was in the same location as the spot found in pregnancy urine. When purified chorionic gonadotrophin was added to human pregnancy urine the filter paper electrophoretic patterns of the urine before and after the addition of the hormone were indistinguishable; this would seem to indicate that the spot due to the protein in pregnancy urine and that due to chorionic gonadotrophin were directly superimposed and therefore probably produced by identical substances.

In addition the urine of one non-pregnant patient who had proteinuria was examined and this specimen was found to contain a complex which migrated toward the anode. However, the urine of two patients who had a high pituitary gonadotrophic titre were tested and these specimens contained a fraction which also migrated toward the cathode. This fraction could easily be confused with chorionic gonadotrophin under the electrophoretic conditions used in the present experiments.

CONCLUSIONS

It is apparent that chorionic gonadotrophin, the placental hormone, migrates toward the cathode at a characteristic rate of speed under conditions described. The proteins usually found in the urine of medical patients with proteinuria migrate toward the anode under similar conditions. This allows for the ready separation and identification of chorionic gonadotrophin from urinary albumin or globulin fractions. The pituitary gonadotropes however are closely related chemically to chorionic gonadotrophin and also migrate

* Follutein Squibb

toward the negative pole. It is therefore possible to confuse chorionic gonadotrophin with the pituitary gonadotrophic hormones under the conditions described unless additional care is used in measuring the speed of migration.

From very preliminary work it appears that a filter paper electrophoretic method may be satisfactorily adapted as a clinical test for pregnancy. As the basis for the test is the electrophoretic identification of chorionic gonadotrophin in the urine it is obvious that there must be an adequate concentration of the hormone present. This would depend both upon the concentration of the urine specimen and the duration of the pregnancy as well as upon other factors concerned with the placental production of the hormone. Such details are being investigated. It appears that high concentrations of pituitary gonadotropes can easily be mistaken for chorionic gonadotrophin under the conditions outlined. This fact must be considered if false positive pregnancy tests are to be avoided.

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PROCEEDINGS OF THE APRIL MEETING OF THE JOHNS HOPKINS MEDICAL SOCIETY

In Association with the Alpha Omega Alpha Fraternity

Hurd Memorial Hall

Monday April 13, 1953

Dr. Jonas Friedenwald: The first order of business will be the report of the Secretary-Treasurer. (The report of the Secretary-Treasurer was accepted).

Dr. Friedenwald: The second order of business concerns the election of officers. (A motion to cast a unanimous vote in favor of the slate of officers was passed. Dr. W. W. Scott was elected President and Dr. Samuel Asper, Secretary-Treasurer.)

Dr. Friedenwald: The rest of the meeting will be given over to the students under the auspices of the Alpha Omega Alpha Fraternity. All presentations will be by medical students who have had one or another interest along research lines in the course of their training here. I am going to ask Dr. Richard Dever to take over the meeting from this point on.

Dr. Dever: I would like to thank the Johns Hopkins Medical Society for turning over this meeting to the Alpha Omega Alpha Fraternity for the presentation of student papers. We would like also to thank those who have shown so much interest in the individual work of these students.

The first paper this evening is by Mr. Frank L. Iber*

SERUM ALKALINE PHOSPHATASE IN THE RAT

The albino rat is widely used for experimental studies on rickets. The experimental disease faithfully reproduces most of the features of the human illness but requires drastic methods for production. Thirty-four rats were permitted to develop rickets on a high calcium and low phosphate diet. Serum levels of alkaline phosphatase in these were compared with levels in thirty-seven similarly fed, but Vitamin D supplemented, animals. No differences were found. Various methods were employed for the determination of serum alkaline phosphatase. Serum levels in individual rats were studied during the course of development of rickets with no significant changes.

Phosphatase activities of serum and of homogenates of epiphysis, kidney, and intestinal mucosa were studied at varying hydrogen ion concentrations. The serum enzyme behaved more like that of intestinal origin than any other. Inhibitor studies employing beryllium, cyanide, fluoride, and alanine, and magnesium activation showed that the serum enzyme acted more like the one of intestinal origin than the enzyme of kidney, liver, or epiphysis. A tentative hypothesis is that the serum enzyme of the rat originates predominantly in intestinal mucosa; bone contributes but slightly to this level. An important difference in rickets in the rat as compared to the human disease is this failure of the serum alkaline phosphatase to rise.

Dr. Leslie Hellerman: One of the very pleasant aspects of teaching in the medical school centers around the interest that students of all 4 classes maintain in the research activities of the several departments in the school. The basic departments are included, and Physiological Chemistry usually attracts a few students who are especially interested in this approach. Mr. Iber is one of the students who has done a little research as time has permitted during

* Henry Strong Denison Scholar in Physiological Chemistry

each of his four years. He has sampled three different problems. His first interest involved the enzymatic hydrolysis of nucleic acids. Next, he became interested in the fate of the oxidation products of Vitamin C, and this year he proposed a study of serum alkaline phosphatase. I am particularly glad that Mr. Iber chose alkaline phosphatase for presentation here tonight. In the first place, it is obvious that Mr. Iber has discussed a problem that has considerable bearing on the use of the rat as a test animal. Moreover, his paper represents a completely independent piece of work. This interest in serum alkaline phosphatase originated with him and he carried it through with very little help indeed. As Mr. Iber has stated, phosphatases are rather unsatisfactory enzymes with which to deal. Some enzymes as you know are highly specific. Urease is said to be absolutely specific for the hydrolysis of urea. Arginase is a rather specific enzyme, but when you deal with phosphatase, you come to realize that the specificity of enzymes generally, in an important sense is a matter of degree rather than an absolute attribute of these catalysts. The enzyme, arginase, for example, is specific in the sense that it is particularly efficient with L(+)arginine and approximately 20% as efficient with D(−)arginine. This enzyme is, however, only 1/1000 as efficient with certain analogues of arginine. In the case of some of the phosphatases, specificity seems to range among various esters of phosphoric acid. To this only rare exceptions are known at present. To determine the origin of rat serum alkaline phosphatase, Mr. Iber has employed several elementary properties of enzymatic action, namely the characteristics of pH-activity curves of enzymes and the use of certain appropriate inhibitors. He has applied these techniques to alkaline phosphatase in preparations of various tissues and has noted comparisons—a procedure of juxtaposition. I think he has done a very nice job. It's a beginning. He has been conservative in his presentation and in my opinion he has accomplished rather convincing definition of his problem.

I hope that all of our students—not only those who have done some research in physiological chemistry—will continue to utilize biochemical and physiological thinking and approaches, where these are applicable, in connection with some of the problems encountered in their continuing medical studies.

Dr. Richard H. Follis, Jr.: I should also like to commend Mr. Iber on his presentation and further commend him on his tenacity in attacking this problem. Mr. Iber was in my teaching group in pathology and one day asked me what I knew about the serum concentration of phosphatase in rachitic rats. I naturally said it was elevated. He said to me, as he has several times since, "Give me a reference." So I went to look at what I had and found I had no reference. I was unable to find anything, so as he had gone to his teacher, I went to my teacher, Dr. Park, and asked him. He made the same reply that I had to Mr. Iber. So I'm afraid I may be responsible for making Mr. Iber a little discouraged about his problem. It's an extremely interesting one. As he has indicated to you this evening, the rat is a peculiar animal in so far as the metabolism of calcium and phosphorus are concerned. Whether the intestine is the main source of phosphatase in the rat is still fully to be proved and I think Mr. Iber will agree. He aims to go further in the work. Dr. Handler and I at Duke some years ago found quite to our surprise that when rats were placed on a diet, calorically deficient, although the bones stopped growing, the serum phosphatase increased. We thought this might be due to some changes in the liver. The importance of phosphatase has been up and down always and particularly so in relation to bones ever since its influence was first thought to be significant.

Dr. A. McGehee Harvey: Have you studied the rat alkaline phosphatase in other conditions known to produce a rise in serum levels and have you investigated the origin of serum alkaline phosphatase in other species?

Mr. Iber: No, I haven't studied the rat under other conditions. It is of interest that liga-

tion of the common bile duct in the rat is reported to produce only mild elevations of the serum alkaline phosphatase. I have studied a few patients with elevated serum alkaline phosphatase and am accumulating evidence that indicates the bone origin and liver excretion theory is inadequate for man.

Dr. Dever: The second paper is by Mr. Daniel S. Bernstein, and Drs. William H. Carnes and L. J. Rather. It will be presented by Mr. Bernstein.

DESOXYRIBOSENUCLEIC ACID CHANGES IN THE RAT LIVER PRODUCED BY THIOACETAMIDE: A MICROPHOTOMETRIC ANALYSIS*

Microspectrophotometry offers unprecedented opportunities for correlating structural changes in the cell with some of its important chemical constituents, notably protein and nucleic acids. This technique has been applied to the analysis of the desoxyribosenucleic acid (DNA) content of normal nuclei of many species, establishing the rule of a direct proportionality between DNA content and the number of chromosomes in the interphase nucleus. Normal variations in DNA content due to mitosis and to polyploidy have been discussed by Swift (Physiol. Zool. 1950, **23,** 169). One of us has reported the induction of cirrhosis in rats with thioacetamide (TA), accompanied by striking cytological alterations in the liver cells (Bull. Johns Hop. Hosp., 1951, **88,** 1). An early increase in nuclear diameters, corresponding to a doubling of mean nuclear volume, suggested an effect on ploidy. This should be reflected in the nuclear DNA content. However, Laird (Fed. Proc. 1952, **11,** 244) has reported that the *average* DNA content of isolated rat liver cells is not altered by thioacetamide, and this has been confirmed by Thomson *et al.* (Biochem. J., 1953, **53,** 460) in short term experiments. Young adult rats have been given 20–40 mg.% TA in the food or drinking water for periods up to 3 months. The relative DNA contents of individual liver cell nuclei have been calculated from measurements of dimension and optical density in Feulgen stains of touch smears and of cells isolated in sucrose and fixed with formaldehyde. At periods approaching 3 months many cells have markedly enlarged nuclei with proportionate increases in DNA content. The values do not conform closely to a geometric series as in the normal liver. The results indicate a disturbance in the process of nuclear division or in the synthesis of DNA.

Dr. Follis: Mr. Bernstein is to be commended for his presentation. I think that all of us must realize the extremely tedious procedures which have to be applied in measuring and getting quantitative results. I think if you have never seen this technique used you don't realize the care and tediousness that accompany the measurements he presented. I think also that it is extremely interesting for us to have seen this procedure demonstrated and the results which may be obtained. Some of you may have attended a meeting of this Society several months ago, when one component of the cell, the mitochondria were discussed. Here one is able to measure another constituent of the cell, the integrity of which has not been changed by cell destruction. I think that it is extremely interesting to do. One gets a good deal of satisfaction, at least I do, and I am sure Mr. Bernstein does in being able to quantitate chemical changes which take place or which are measurable in cells. We get a little tired I think of gauging normal changes and pathological changes, 1 plus, 2 plus, 3 plus. If one can actually demonstrate chemical differences by this technique, I think it affords you a great deal of satisfaction. I might indicate to you that the amounts of material with which Mr. Bernstein has been measuring and which he has presented are relatively very, very minute. Dr. Carnes with whom Mr. Bernstein did this work in California, last

* The Department of Pathology, Stanford University, School of Medicine. (Assisted by a Grant-in-Aid of the U. S. Public Health Service.)

summer, wrote me that one is able to measure within an error of 10% the DNA content of a liver nucleus, whose total content is 5×10^{-9} milligram. And that comes out to 5/billionths of a milligram. So one is getting down to real micro histochemistry by this particular technique.

Dr. Hellerman: I presume that this effect of thioacetamide was a highly specific one. I wonder whether such a substance as thiouracil, when used in large amounts, has been observed to induce anything similar to this.

Mr. Bernstein: I think that I can answer that, Dr. Hellerman. Studies were started in Washington to investigate the toxicity of thiouracil and thiourea, and it was found that thiourea produced hepatic cell adenomas in rats with great frequency. Thioacetamide, which is in this family of drugs, produced cirrhosis, and Dr. Rather was stimulated to study the nuclear changes and other pathological changes found in this condition.

Dr. Dever: The next paper is by Mr. Malcolm Stoler.

SUSCEPTIBILITY OF A HUMAN FIBROSARCOMA LINE TO POLIOMYELITIS VIRUS

The recent demonstration by Dr. Enders that poliomyelitis virus will grow in and destroy human and monkey non-neural tissues has stimulated a great deal of interest and work on this virus in the tissue culture field. A myriad of papers on the growth of the virus has ensued, such that the cultivation of poliomyelitis virus has been convincingly demonstrated in a number of human embryonic tissues, monkey testicular tissue, and human foreskin. However, all these studies were done on primary explants, which consist of mixed cultures of cells. Uniform cell lines are obviously more desirable in the study of the host-parasite relationship under controlled conditions with as many variable factors as possible eliminated or controlled.

AFi, a human fibrosarcoma line which has been in continuous cultivation in Dr. Gey's laboratory since 1935, proved to be susceptible to the virus. The method of cultivation was as follows: small pieces of tissue, 2 to 6 in number, approximately 1 mm.^{3} were placed in sterile test tubes. Ten drops of a 25% chicken serum plus 100 units of penicillin and 10 units of streptomycin were added. The tubes were then stoppered and incubated in a revolving drum at 34°C. Daily recordings were made of the growth of the cells. The infecting medium consisted of a homogenate of the brains of mice in various stages of paralysis after being inoculated with the Lansing strain of poliomyelitis virus. The tubes were checked daily for rounding up of the cells, increased granularity, fraying of the cell membranes and/or actual necrosis of the sarcoma cells.

To confirm the susceptibility of AFi and its ability to sustain propagation of the virus, ten serial passages of the virus in the cell line were effected. In all passages, the infected cells were uniformly destroyed at the end of 72 hours. In all cases, the estimated percentage destruction was greater than 60% and in almost all cases varied from 80 to 100%, as compared to about 10% destruction in the controls. Within the first 24 hours after infection, evidence of the viral effect became apparent in the form of increased accumulation of droplets and granules and a fraying of the edges of some of the cells. This progressed to the point where a large proportion of the cells became rounded, dark, granular, and irregular in outline. After about 60 hours, most of the cells were definitely necrotic, with ruptured cell membranes, cytolyzed fragments and debris scattered over the colony and tube. Motion pictures, carried out by Dr. Gey and Mr. Sapranauskas of suitable cultures showed that the destructive processes progressing from the first stages of irregular fraying of the edges of the cell to an advanced degree of necrosis takes as long as 12 hours. The titers of virus in the tissue culture fluids remained approximately the same for all passages.

To prove that the cell-destroying factor was actually Lansing polio virus and not some

bacterial contaminant or toxic substance, neutralization studies were carried out using immune monkey serum from monkeys previously infected with the Lansing strain. Normal monkey serum was used as a control. The results of the experiment were as follows: at the end of 48 hours, the cells in the control tubes were completely destroyed. The cultures containing the immune serum, on the other hand, were protected at all dilutions of the immune serum (1/10, 1/50, and 1/250) and showed at the end of observation abundant cell growth and migration without evidence of virus effect.

Thus, the propagation of poliomyelitis virus has been demonstrated in this susceptible human tumor cell line. Evidence for this rests on its uniform destructive effect on all passages, on the recovery of the virus in mice, and the neutralization of the destructive effect by immune monkey sera. With this and other uniform cell lines, the host-parasite relationship can be more adequately studied, the whole interchange and effects of the virus and cell on each other being stripped, as it were, to its bare essentials. Cell specificity and immunity to viral attack, viral effects on cell metabolism, etc., can be studied under more controlled conditions in a single cell line.

Dr. Frederik B. Bang: I would like to commend Mr. Stoler. Frequently in the complexities of research, we forget individuals and their attempt to study individual problems. Mr. Stoler's problem involved two laboratories, that of Dr. Gey's and mine. I think that it is fortunate that he has had the tenacity to take one problem and work with it in two separate laboratories.

In a way, one can classify in three ways the methods of tissue culture used in the study of viruses. The usual method is to add virus to a mixed culture of tissue cells and see whether or not the virus grows. The second method, which is clearly demonstrated here, is the study of the effect of virus on certain pure tissue cell lines that present a high degree of uniformity. Mr. Stoler concluded clearly how some possible uses of this method could be brought about. The third method has not been touched but perhaps will be some day. It is the study of the effect of virus on tissue cultures of organoids taken from the original host, with the object of determining the specificities of virus effect on the organ.

I think that this presentation also clearly demonstrated how Mr. Stoler, given a problem and using the facilities of the two laboratories and the degree of tenacity and intelligence shown here, was able within a relatively short period of time, to uncover new approaches to certain problems. This work was done completely during the time period of last summer.

The fourth and last speaker of the evening will be Mr. James Messer.

CHIARI SYNDROME: THROMBOSIS OF HEPATIC VEINS*

The appearance at this hospital of a case of hepatic vein thrombosis (as determined by laparotomy and liver biopsy) has provided an opportunity to review the literature concerning this subject. In most of the 106 cases collected from the medical literature the ostia of the hepatic veins have been the initial site of block. Depending upon the duration of life after thrombosis, the clots may undergo organization and canalization. The average age incidence is 34. Predisposing factors include polycythemia vera, block by gummatous material, phlebitis of hepatic veins, peptic ulcer, hepatic hyperplasia, and encroachment of hepatic veins by primary or metastatic new growth.

Abdominal pain, variable in character, is the initial and most constant clinical feature and is caused by stretching of the capsule following rapid engorgement of the liver. Ascites, which characteristically recurs extremely soon after paracentesis, rapid hepatic enlargement, and liver tenderness are the next most common features. The establishment of venous

* This paper appears in full in *Tufts Medical Journal*, May, 1953.

collaterals occurs later in the course of the disease and is especially diagnostic in that the distribution is limited to the lower thoracic and upper abdominal walls. Edema of the lower extremities and vomiting occur in 50% and 25% of the cases, respectively. Jaundice is usually absent. Liver function studies usually remain normal with the exception of elevated prothrombin time. The differential diagnosis includes portal vein thrombosis, cirrhosis, and right heart failure.

The prognosis is unfavorable. The patient may survive only a few weeks and usually lives less than 6 months. In a small group of patients the disease may last from 10 to 28 years, and occasionally carcinoma of the liver will finally occur. The treatment is unsatisfactory. Evaluation of anticoagulant or trypsin therapy is not possible at the present time. Surgical procedures, including omentopexy, have not been beneficial, however shunting operations such as portocaval anastomosis have not as yet been tried.

Thrombosis of hepatic veins is unusual, even as an incidental finding at autopsy. The condition was found in 5 of nearly 12,000 autopsies done at Stanford, and the Mayo Clinic has reported 20 cases. In reviewing over 21,000 autopsies done at the Johns Hopkins Hospital, we were able to find only 17 cases; one of these cases was associated with congenital narrowing of the hepatic veins, one with trauma, six with biliary and/or hepatic infection, five with neoplastic lesions, one with multiple gastric ulcers, one with severe diarrhea and acidosis, one with hemosiderosis following multiple blood transfusions, and one with lobular pneumonia and pericarditis.

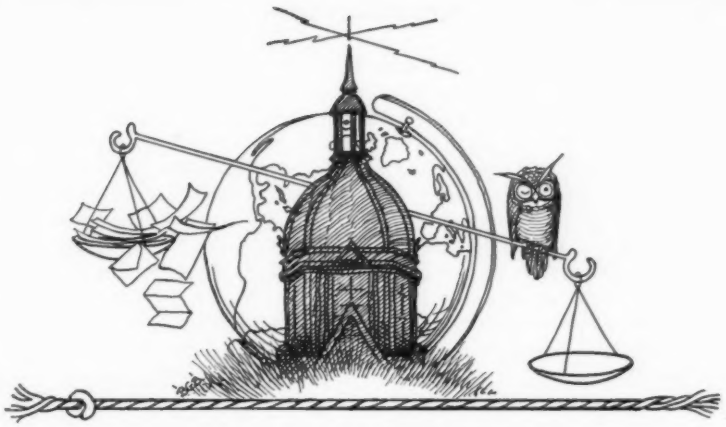
Dr. Harvey: I would like to comment on this paper. It gives me pleasure to hear a clinical paper of this type presented at this meeting. Several years ago, when Dr. Follis and I had the privilege of acting as officers of this Society, these meetings under the sponsorship of the A.O.A., where students presented their own original work, were begun. It was part of our original idea that not only would work done in the laboratories be presented but a program of clinical observations of interest would also be a part. This to my knowledge is the first time that part of the concept has been carried out and a clinical paper has been presented. I think that all of the papers this evening were very good, and beautifully done. All are examples of very excellent work on the part of these students. The reason that I take particular pleasure in this last paper is because I think that many students tend to forget the importance of clinical observations and what may come out of them in the way of new knowledge about the natural evolution of diseases as we see them today in our practice. It is not everyone who has the opportunity or even the ability to go into the laboratories and use many of the complex techniques for the study of diseases that are used today. However, all of us have the opportunity to make some contribution to the total sum of medical knowledge even though it may be small. We gain so much from the material that has been gathered over many, many years by numerous observations of a tremendous number of people. This body of information represents what we are taught in school, and I think that it is of tremendous importance to try and stimulate every student who goes out of this school to feel that in his lifetime he should maintain an attitude of curiosity for the things he is going to see in medicine and hope that he will make some original contribution. Now as a matter of fact the contributions that deal with clinical observations are among the most important that have been made in the total field of medicine. One only has to think about the beautiful study of the introduction of digitalis into medicine, and contributions of people like MacKenzie. One can cite numerous examples of important discoveries made in this institution by people during student days. The first recognition of the relationship of eosinophilia to hookworm disease was made by Dr. Thomas Brown in this institute when he was still a medical student. Another is the observation made by Dr. Ralph Major who first described clubbing of fingers in bacterial endocarditis. Dr. Albright first described the syndrome of third nerve palsy

with subarachnoid hemorrhage with congenital aneurysm of the circle of Willis when an assistant resident in this hospital. I think that in encouraging original investigation it is important to point this out. All of us no matter what we do in medicine, or where we practice, can make important contributions of an original nature both in the laboratory and in the day to day observation of patients.

As you know the problem of intravascular thrombosis is one which receives a great deal of attention and much work has been devoted to it by talented people. Still we have no good idea of its pathogenesis. Although this syndrome is of rather rare occurrence, perhaps it has some unique feature which if understood would give us new knowledge of venous disease with thrombosis.

Dr. Friedenwald: I'd like to thank the Alpha Omega Alpha Fraternity for the remarkably good program they have submitted to us at this time in closing this season's meetings of the Medical Society. I would like to say for Dr. Wagley and myself; we deeply appreciate the honor you gave us in making us officers of the Society. We are deeply grateful to all of those who contributed to the programs during this year.

Motion was made and seconded to adjourn meeting. Meeting was adjourned at 10 P.M.



BOOK REVIEWS

(These reviews represent the individual opinions of the reviewers and not necessarily those of the members of the Editorial Board of the Bulletin)

Poliomyelitis. Papers and Discussions Presented at the Second International Poliomyelitis Conference. Compiled and Edited for the International Poliomyelitis Congress. 555 pp., $7.50. J. B. Lippincott Co., Philadelphia, Pa.

This book contains the papers and exhibits presented to the Second International Poliomyelitis Conferences in Copenhagen in September, 1951.

It is divided into eight sections. The first section presents basic concepts of virus and its reaction with host cells. The second is devoted to growth of poliomyelitis virus and the pathology and pathophysiology of poliomyelitis. The third section covers the Coxsackie group of viruses. The fourth is devoted to the clinical and laboratory aspects of differential diagnosis. The fifth section deals with medical and orthopedic treatment of poliomyelitis. The sixth covers immunity and resistance. The seventh is devoted to the ecology of poliomyelitis. The last section contains exhibits illustrating points presented in the preceding sections.

The book is authoritative, comprehensive, well printed and well illustrated. Each article is concerned with a significant contribution to knowledge of poliomyelitis and is written by the man who did the work. The articles in each of the first seven sections are followed by stimulating discussion by qualified men.

The book is concerned with the facts fairly definitely established about poliomyelitis by September, 1951. Therefore, though it deals with a field in which new knowledge has been added since publication, it is not out of date. Rather, it serves as a good source of background material necessary for comprehension of the new knowledge. The book, therefore, can be highly recommended as a reference volume.

THOMAS E. VAN METRE, JR.

An Atlas of Surgical Exposures of the Extremities. By Sam W. Banks and Harold Laufman. 391 pp., $15.00. W. B. Saunders Co., Philadelphia, Pa.

This is a handsomely produced Anatomical Surgical Atlas with lavish use of fine plates

and a minimum of text. It will be a godsend to the hospital resident who is being called on frequently to meet at a moment's notice operative problems new to him, and to the intern or medical student to clarify procedures he has been watching someone else do with too little demonstration of the anatomy encountered. The general surgeon who is called from his familiar visceral latitudes to the outlying regions of the extremities will find it a ready and clear and useful "road map" to follow. Even the seasoned orthopedic surgeon will find it a valuable aid to refresh the anatomy of some operation he has not undertaken recently or in selecting the easiest approach to a particularly tough problem. The authors have been most meticulous in attaining accuracy by their method of dissection and photography of each step of the procedure as described in their preface. They have been almost as carefully objective in the text, making it clear, simple and factual and only on one occasion expressing editorial comment. Also they are very laconic in their heading of "Indications". This resolute objectivity is wise and is successful in making the volume extremely usable. It does, however, rob the book of the stimulation that comes from explanation and discussion in the text, such as Henry gives us in his somewhat controversial book on the same subject. This objectivity has also had its like effect on the artistic quality of the illustrations which are definitely redolent of formalin rather than of ether. The artist has been most faithful to her material, but as a surgeon we miss the "living" quality one sees so admirably conveyed by illustrations taken direct from the operating room. No doubt the authors are equally aware of these non-essentials and deliberately sacrificed them in order to give us what they have—an extremely clear, accurate, concise, graphic and usable surgical atlas. We owe them a debt of gratitude and appreciation for a long, hard job excellently accomplished. It is a most useful book to any surgeon to have close at hand.

ROBERT W. JOHNSON, JR.

Visual Anatomy: Thorax and Abdomen. By Sydney M. Friedman. 212 pp., $10.50. Charles C Thomas, Springfield, Illinois.

This book represents the second of a series of anatomical texts by this author on what he has titled, *Visual Anatomy*, the previous volume pertaining to the anatomy of the head and neck. The organization and manner of presentation is the same in both books, the emphasis being placed entirely on diagrams and sketches drawn by the author himself, each accompanied by a brief anatomical description. The author has taken the separate anatomical regions of the thorax and abdomen, described them in terms of their basic structural framework and relations, and then built on them, so to speak, by a series of diagrams the various contiguous structures. Such an approach would seem a definite aid in the understanding and review of this subject.

For the student or practitioner desirous of a quick general anatomical review of the thorax and abdomen, this book would seem well adapted. For the surgeon it would be of little value in that it is far lacking in sufficient detail, description and illustration, especially of those areas of greatest surgical importance. There is very little clinical correlation of described structures and relations. This book cannot be regarded as a basic or supplemental anatomical text, the author never intending it as such, but instead might more properly be described as anatomy in its simplest terms.

Dr. Friedman's stated purpose of this book as being "designed as a review for the student" would seem to have been well fulfilled.

VERNE E. CHANEY, JR.
I. RIDGEWAY TRIMBLE

Infant and Maternal Care in New York City. A Study of Hospital Facilities. Sponsored by the Committee on Public Health Relations, The New York Academy of Medicine. E. H. L. Corwin, General Director of Study. 188 pp., $3.50. Columbia University Press, New York.

This study of hospital facilities for infant and maternal care in New York City was sponsored by the Committee on Public Health Relations of the New York Academy of Medicine. A team consisting of an obstetrician, a nurse with pediatric training and a pediatrician visited 104 of the 107 hospitals with maternity services in New York City. The New York City Department of Health assisted in the study throughout. The result is a meticulous evaluation of maternity and newborn infant hospital services in that community.

Use was made of a detailed "inventory form" listing more than 250 specific items, each of which was checked either by the observing team or by questionnaire. Since less than one per cent of births in New York City occur outside hospitals, the findings can be considered descriptive of total maternity and newborn services in that city. Inventory "scores" for individual hospitals bore a positive relationship to Approval by the American College of Surgeons, i.e. fully approved hospitals averaged a rating of 72, while those lacking approval averaged a score of 38.

Some of the more striking findings include the following: less than half of the maternity services could be rated as "good" and many did not meet even minimum standards for adequate care. Major deficiencies consisted of lack of necessary equipment, practices so faulty as to constitute a definite hazard to mothers and their babies, widespread shortage of personnel, lack of qualified obstetricians, pediatricians, and nurses. Often there was no pediatric supervision in newborn nurseries except in case of illness. In many instances qualified personnel had appointments "on paper" with actual work being done by persons lacking proper qualification. Maternity services of less than 20 beds appeared definitely inferior to larger services.

New York City has the lowest infant mortality rate of any city in this country of more than one million population. The above shortcomings must be viewed in this light and furnish food for thought to anyone interested in the broad aspects of infant and maternal care anywhere. The method of study and the "inventory form" will prove of great value to anyone undertaking an appraisal of hospital maternity and newborn services and, in fact, any type of hospital service.

J. WHITRIDGE, JR.

BOOKS RECEIVED FOR REVIEW

Clinical Diagnosis by Laboratory Methods, 12th edition. By James Campbell Todd, Arthur Hawley Sanford and Benjamin B. Wells. 998 pp., $8.50. *W. B. Saunders Co., Philadelphia, Pa.*

Clinical Endocrinology. Vols. I and II. By Lewis M. Hurxthal and Natalija Musulin. 1599 pp., $24.00. *J. B. Lippincott Co., Philadelphia, Pa.*

The Conception of Disease. Its History, Its Versions and Its Nature. By Walther Riese. 120 pp., $3.75. *Philosophical Library, New York.*

Encyclopedia of Aberrations. Edited by Edward Podolsky. With a Foreword by Alexandra Adler. 550 pp., $10.00. *Philosophical Library, New York.*

Functional and Surgical Anatomy of the Hand. By Emanuel B. Kaplan. 288 pp., $10.00. *J. B. Lippincott Co., Philadelphia, Pa.*

Microbiology and Human Progress. By Madeleine Parker Grant. 718 pp., $6.75. *Rinehart & Co., Inc., New York.*

Operative Gynecology. 2nd edition. By Richard W. Te Linde. 902 pp., $20.00. *J. B. Lippincott Co., Philadelphia, Pa.*

Physical Examination and the Surgical Patient. By J. Englebert Dunphy and Thomas W. Botsford. 326 pp., $7.50. *W. B. Saunders Co., Philadelphia, Pa.*

Physiological Foundations of Neurology and Psychiatry. By Ernst Gellhorn. 556 pp., $8.50. *University of Minnesota Press, Minneapolis, Minnesota.*

Problems der Morphologie, Cytochemie und Wuchsform des Tuberkuloseerregers. By F. J. Bassermann. 98 pp., kart. DM 7.80. *Georg Thieme Verlag, Stuttgart, Germany.*

Symptom und Kausalität. Grundfragen der Neurologie und Psychiatrie. By F. Llavero. 251 pp., Ganzleinen DM 33. *Georg Thieme Verlag, Stuttgart, Germany.*